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Interaction of *Trypanosoma cruzi* with macrophages

Involvement of surface galactose and N-acetyl-D-galactosamine residues on the recognition process

TANIA C. ARAÚJO-JORGE^{1, 2}, W. DE SOUZA²

Summary

The ability of the surface galactose (Gal)/N-acetyl-D-galactosamine (GalNAc) receptor of mouse peritoneal macrophages to recognize bloodstream trypomastigotes of *Trypanosoma cruzi* was examined. The parasite's uptake is improved by its desialylation and impaired by its treatment with Gal or GalNAc-binding lectins. Further incubation of asialoparasites with lectins for Gal-blockage (PNA and RCA I) reverses, in a dose-dependent way, 35–80% of the neuraminidase effect on the endocytosis of *T. cruzi*. Similar effects were observed when lectins for GalNAc-blockage (PHA, WPA and DBA) were used. Asialoerythrocytes or galactosyl-oligosaccharides added during the parasite-cell interaction assays, also competed with the normal or desialylated trypomastigotes for receptors on the host cell surface, inhibiting their uptake and reversing the effect of neuraminidase. Although indirect, these results are strongly suggestive that the Gal/GalNAc recognition system of the macrophages is involved in the interiorization of *T. cruzi*.

Key words: *Trypanosoma cruzi*; macrophage; sialic acid; galactose; receptor-mediated phagocytosis.

Introduction

The invasion of mammalian host cells by trypomastigote forms of *Trypanosoma cruzi*, the aetiological agent of Chagas' disease, is a crucial step in the parasite's life cycle. Polymorphonuclear leukocytes and macrophages are prob-

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ably among the first cells of the vertebrate host with which *T. cruzi* interacts in vivo (Deutschländer et al., 1978). Macrophages support the parasite's intracellular proliferation for some time, until an effective immune response is elicited and a specific stimulation makes the macrophage competent to destroy the trypomastigotes (Hudson and Britten, 1985).

The interaction of *T. cruzi* with macrophages has been extensively studied in the last few years. Carbohydrate moieties from glycoproteins and/or glycolipids were shown to participate in the process of *T. cruzi*-macrophage recognition (Araújo-Jorge and De Souza, 1984, 1986; Villalta and Kierszenbaum, 1983, 1984, 1985). Sialic acid residues exposed on the parasite's surface (Schauer et al., 1983; Souto Padrón and De Souza, 1985) seem to make the interiorization of *T. cruzi* by the macrophages more difficult, as treatment of the parasite with neuraminidase (Araújo-Jorge and De Souza, 1984), cationized ferritin (Meirelles et al., 1984) or *Limulus polyphemus* agglutinin (Araújo-Jorge and De Souza, 1986) facilitates its uptake by these phagocytes. Otherwise, galactosyl (Gal) and N-acetyl-galactosaminyl (GalNAc) residues exposed on the parasites surface seem to be necessary for the adhesion of trypomastigotes to the macrophages and their interiorization, since treatment of parasites with lectins specific to these carbohydrates inhibits the invasion of *T. cruzi* (Araújo-Jorge and De Souza, 1986). Gal and GalNAc are sugars commonly exposed after desialylation of sialoglycoconjugates (Suzuki, 1976). Moreover, a neuraminidase activity, specially expressed in trypomastigotes of *T. cruzi*, was described (Pereira, 1983) and its possible role in cell invasion and in the pathogenesis of experimental Chagas' disease has been proposed (Csete et al., 1985; Pereira and Hoff, 1986; Libby et al., 1986).

A lectin-like protein that recognizes Gal and GalNAc (Gal/GalNAc receptor = R) is present on the surface of macrophages (Nagamura and Kolb, 1980; Kolb et al., 1980), and mediates the binding and interiorization of macromolecules and cells, which have these sugars terminally exposed (Jancik et al., 1978; Kuster and Schauer, 1981; Kolb-Bachofen et al., 1984). The binding and internalization characteristics of this receptor-mediated endocytic system is known in macrophages and hepatocytes (Lee et al., 1982; Kolb et al., 1980).

In the present work we examined the ability of mouse peritoneal macrophages to recognize bloodstream trypomastigotes of *T. cruzi* sequentially treated with neuraminidase and lectins that block D-Gal and/or D-GalNAc residues. We also analyzed the possible competition of asialoerythrocytes and galactosyl-terminal oligosaccharides with normal or desialylated trypomastigotes for a Gal/GalNAc-R on the host cells. Our results suggest the involvement of the Gal/GalNAc recognition system of the macrophages in the uptake of *T. cruzi*.

Materials and Methods

T. cruzi-macrophage interaction. Macrophages and parasites were obtained according to Araújo-Jorge and De Souza (1986). Monolayers of normal peritoneal mouse macrophages were cultivated during 24 h in 199 medium supplemented with 10% fetal calf serum. The Y strain of *T. cruzi* was used, and bloodstream trypomastigotes were purified in a metrizamide gradient (Loures et al., 1980). The parasites were washed twice in cold PBS supplemented with 0.5% bovine serum albumin (PBS-BSA) in Eppendorf microfuge tubes. Macrophages were washed in saline and incubated in the presence of normal, desialylated or neuraminidase-lectin-treated *T. cruzi* trypomastigotes (10:1 parasite/macrophage ratio) in 199 medium without serum for 1 h at 37°C in a humid 5% CO₂ atmosphere. In some experiments, α -lactose, β -lactose or rafinose (20 mM, Sigma Chem. Co.) were added during the interaction period.

Pre-treatment of parasites with neuraminidase and lectins. Parasites were desialylated by incubation in 0.25 U/ml neuraminidase from *Clostridium perfringens* (Sigma type X), dissolved in Ringer solution, pH 6.4, for 45 min at 37°C, washed 3 times in cold PBS-BSA and further incubated for 20–30 min at 4°C (Araújo-Jorge and De Souza, 1986) in one of the following lectin solutions: RCA-I (from *Ricinus communis* I), PNA (from *Arachis hypogaea*), PHA (from *Phaseolus vulgaris*, type III), WFA (from *Wistaria floribunda*) or DBA (from *Dolichos biflorus*). All lectins and carbohydrates were obtained from Sigma Chem. Co. As neuraminidase removes sialic acid, thus exposing new carbohydrate residues, agglutination tests were done to determine sub-agglutinating concentrations of the lectins (table 1). Under these conditions parasite motility was not altered. After the incubation with lectins the parasites were washed 3 times in cold PBS-BSA and counted in a Neubauer chamber to calculate and adjust the parasite-cell ratio. Morphology and motility of parasites were not affected by the experimental treatments, as shown by observation under a phase contrast microscope. Controls were done by adding 50 mM of the specific inhibitory sugar during the incubation with lectins (β -D-galactose for RCA I and PNA and N-acetyl-D-galactosamine for PHA, WFA or DBA) (Nicolson, 1974; Alroy et al., 1984).

Asialoerythrocytes. Recently collected human erythrocytes were washed twice in PBS and adjusted to 5% (v/v). Neuraminidase treatment was performed as described for the parasites, and the degree of sialic acid removal was controlled by titration in agglutination tests with the lectin, PNA. Normal erythrocytes do not agglutinate with 1000 μ g/ml PNA while neuraminidase-treated erythrocytes did so even with 0.2 μ g/ml. Suspensions of 0.5 and 2.5% (7 or 35×10^6 cells/ml, respectively) were prepared and used in the parasite-macrophage interaction assays as ligand-competing particles for the Gal/GalNAc-R. The phagocytosis of asialoerythrocytes was prevented by 25 mM β -lactose (Nagamura and Kolb, 1980) or by the 50 mM level used for control in the present study (data not shown).

Evaluation of results. After *T. cruzi*-macrophage interaction the coverslips were washed 3 times in PBS, fixed in Bouin and stained with Giemsa. The percentage of infected macrophages, the mean number of intracellular parasites per infected macrophage and the number of parasites/100 macrophages (endocytic index) were calculated (Araújo-Jorge and De Souza, 1984, 1986). In each experiment the endocytic indices were normalized by taking the values obtained for the control as 100 and the results expressed as variation related to the control. Statistical analysis was performed using the F-test to the log of the endocytic indices obtained. The results were expressed as means \pm standard deviations.

Results

When the interaction of *T. cruzi* with macrophages was performed at 37°C, the parasites were rapidly interiorized and after 1 h 17–33% of infected macrophages could be seen in the cultures, with 1 or 2 intracellular parasites in the majority of the infected macrophages. The endocytic index on controls corre-

sponded to parasites/100 macrophages and varied from 23 to 55 (Araújo-Jorge and De Souza, 1986).

Sequential treatment with neuraminidase and lectins

As previously described (Araújo-Jorge and De Souza, 1984), treatment of bloodstream trypomastigotes with neuraminidase improved invasion of the macrophages (Fig. 1). Further incubation of desialylated parasites with lectins that block Gal residues (PNA and RCA I) reversed the effect of neuraminidase on ingestion of *T. cruzi* by macrophages by 35–80% (Fig. 1, Table 1). This effect was dependent on the concentration of the lectin. For instance, at low concentration (0.5 µg/ml) PNA did not interfere with the ingestion of neuraminidase-treated parasites by macrophages. At a higher concentration (10 µg/ml), however, an inhibition of about 60% was observed (Fig. 1). Normal trypomastigotes, which did not agglutinate even with high doses of PNA (1000 µg/ml), were susceptible to agglutination after desialylation when incubated in the presence of 15 µg/ml PNA (Table 1). Similarly, the minimal concentration needed to agglutinate normal parasites with RCA I was 62.5 µg/ml, and dropped to 12.5 µg/ml after treatment with neuraminidase (Table 1).

Treatment of desialylated trypomastigotes with lectins that bind to GalNAc residues (PHA, WFA and DBA) also reversed the neuraminidase effect upon the interiorization of *T. cruzi* by macrophages (Fig. 1, Table 1). The treatment of normal trypomastigotes with PHA and WFA could inhibit *T. cruzi*-macrophage interaction (Fig. 1, Table 1). These parasites could be agglutinated only with 50 or 300 µg/ml PHA or WFA, respectively (Table 1). After desialylation, these same lectins agglutinated the parasites at lower concentrations, 10 and 0.6 µg/ml, respectively (Table 1). The susceptibility to agglutination by WFA was so much enhanced, that only 0.5 µg/ml lectin treatment on the desialylated parasites could be used in the interaction assay. In this case, there was still a 30% reversal of the neuraminidase effect upon the endocytic index (Fig. 1, Table 1). DBA lectin had no effect on normal trypomastigotes, but could agglutinate desialylated trypomastigotes at a concentration of 15 µg/ml, and reversed the effect of neuraminidase on *T. cruzi* interiorization.

Competition of asialoerythrocytes and galactosyl-saccharides with desialylated parasites

Normal or neuraminidase-treated trypomastigotes were allowed to interact with macrophages in the presence of 20 mM α -lactose (β -galactopyranosyl-1-4- α -glucopyranosyl), β -lactose (β -galactopyranosyl-1-4- β -glucopyranosyl) or raffinose (α -galactopyranosyl-1-6- α -glucopyranosyl-1-4- β -fructofuranosyl) (Fig. 2). α - and β -lactose did not affect the endocytosis of normal trypomastigotes, but reversed the neuraminidase effect upon the endocytic index, β -lactose being the most effective (58–91% reversion). Addition of asialoerythrocytes inhibited the uptake of normal trypomastigotes in a dose-dependent way up to 95% (Fig. 2).

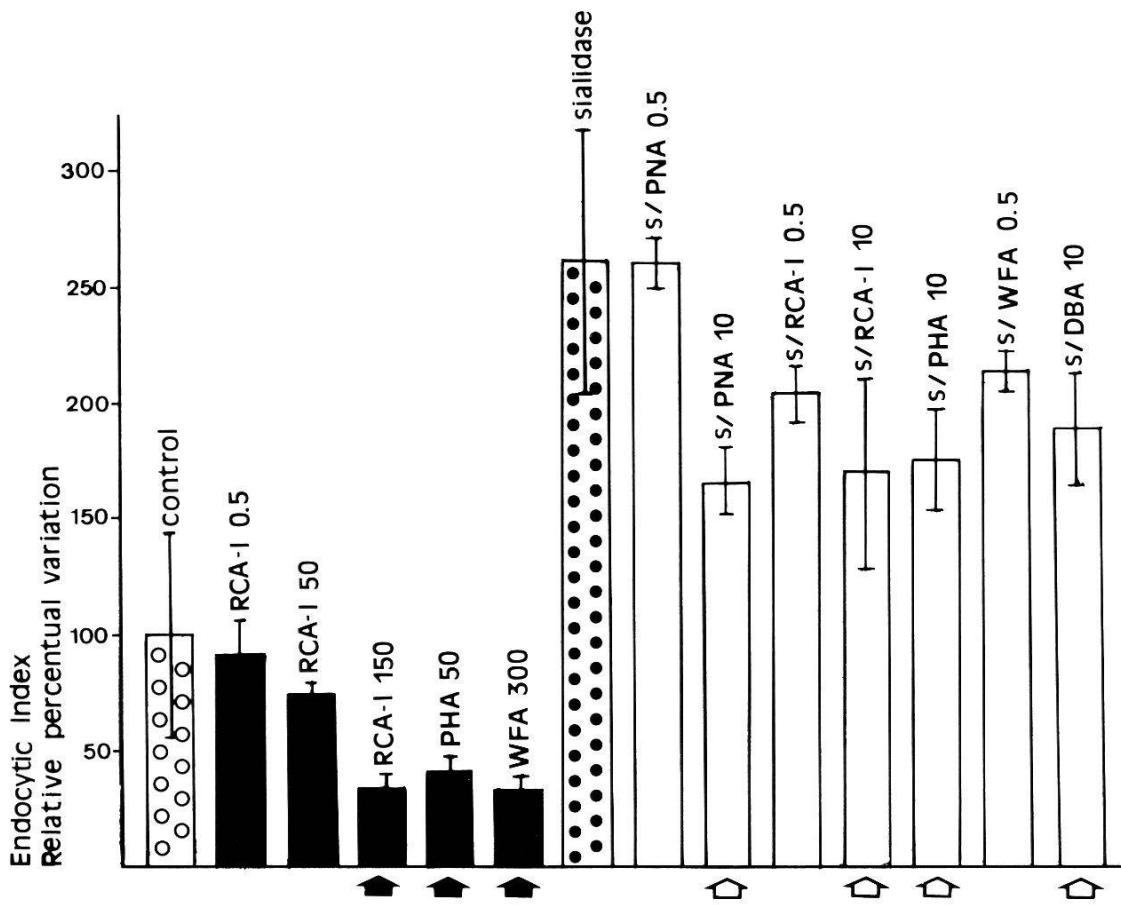


Fig. 1. Effect of the treatment of *Trypanosoma cruzi* bloodstream trypomastigotes with neuraminidase, lectins, or both agents in sequence, on their endocytosis by normal mouse peritoneal macrophages after 1 h of interaction. Numbers right to lectin symbols indicate the concentrations (in $\mu\text{g}/\text{ml}$) used to treat the parasites. S/lectin indicates lectin treatment after desialylation of the parasites. Arrows indicate statistically significant differences as compared to controls.

Discussion

The surface of macrophages has multiple recognition systems that mediate endocytosis (Adams and Hamilton, 1984). Some of these are restricted to specific phagocytes as Fc or Cb3 receptors, while others are also found in non-specific phagocytic cells. Lectin-like receptors for carbohydrates (Mannose/N-acetyl-D-glucosamine/fucose receptor, abbreviated as Man-R, and the Galactose/N-acetyl-D-galactosamine receptor, abbreviated as Gal/GalNAc-R) or the receptors for the α_2 -macroglobulin-protease complexes are also present in hepatocytes, fibroblasts or muscle cells (Neufeld and Ashwell, 1979; Kolb-Bachofen et al., 1984; Roff et al., 1983; Straus, 1983; Tagerud and Libelius, 1985; Hanover et al., 1983).

The interiorization of *T. cruzi* by macrophages can be stimulated by immunoglobulin and complement factors (Nogueira et al., 1980) but these opsonins are not essential for invasion. Other ligand-receptor interactions must take place since the parasite-cell assays can be successful in vitro in the absence

Table 1. Effect of incubation of control or neuraminidase-treated bloodstream trypomastigote forms of *Trypanosoma cruzi* in the presence of lectins on parasite agglutination and ingestion by macrophages

Sugar specificity	Lectin	Neuraminidase treatment	Minimal concentration ($\mu\text{g}/\text{ml}$) required to agglutinate	Effect on the endocytic index (using a sub-agglutinating concentration)
β -D-galactose	PNA	no	1000	Inhibition*: no
		yes	15	Reversion**: 60%
	RCA I	no	62.5	Inhibition: 65%
		yes	12.5	Reversion: 22–80%
N-acetyl-D-galactosamine	PHA	no	50	Inhibition: 60%
		yes	10	Reversion: 40–60%
	WFA	no	300	Inhibition: 70%
		yes	0.62	Reversion: 25–35%
	DBA	no	1000	Inhibition: no
		yes	15	Reversion: 45%

* Inhibition of the endocytosis of normal parasites.

** Reversion of neuraminidase effect on the endocytosis of parasites.

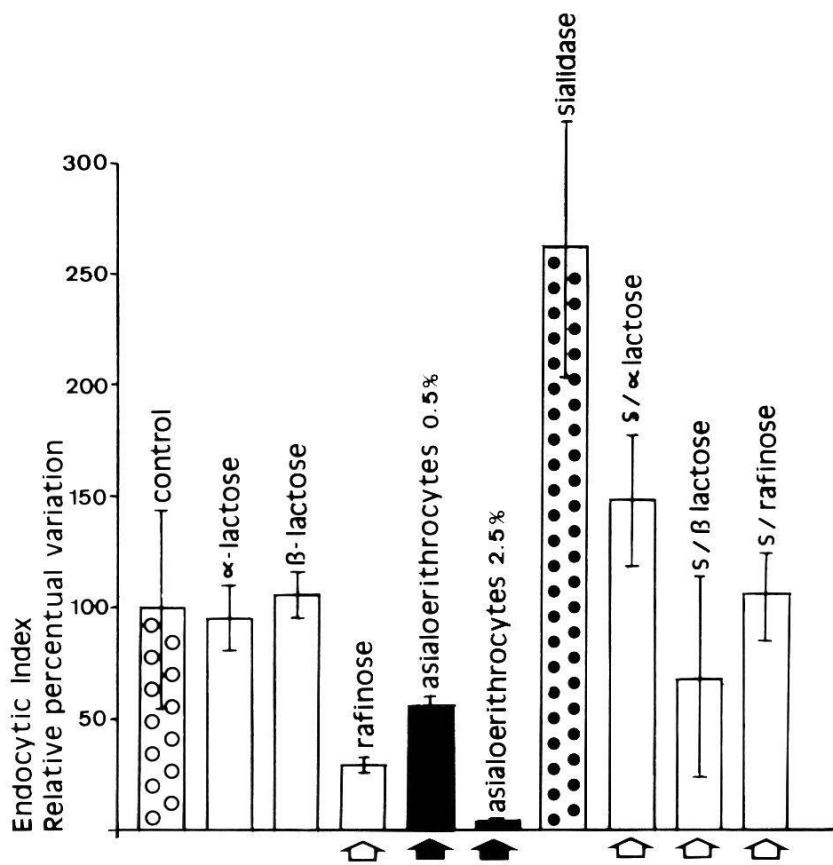


Fig. 2. Effect of the addition of galactosyl-oligosaccharides and of asialoerythrocytes during 1 h of interaction between normal or desialylated parasites and macrophages. Arrows indicate statistically significant differences as compared to controls.

of serum (Araújo-Jorge and De Souza, 1984, 1986). The involvement of carbohydrate residues on *T. cruzi*-macrophage interaction has been suggested by many experiments (Araújo-Jorge and De Souza, 1984, 1986; Villalta and Kierzenbaum, 1983, 1984, 1985; Meirelles et al., 1984).

The presence of Gal and GalNAc during the interaction assays (Araújo-Jorge and De Souza, 1984), as well as treatment of *T. cruzi* with lectins that bind to these sugars (Araújo-Jorge and De Souza, 1986) impaired parasite invasion. The assumption of participation of the macrophage Gal GalNAc-R in this situation was logical and tempting, and the effect of neuraminidase (Araújo-Jorge and De Souza, 1986), enhancing the phagocytosis of trypomastigote forms of *T. cruzi*, also was consistent with this hypothesis.

In the present study we have shown that, after removal of sialic acid residues by neuraminidase, the trypomastigotes became more susceptible to agglutination by lectins that bind to newly exposed Gal and GalNAc residues (PNA or RCA I, and PHA, WFA or DBA, respectively). The blockage of these residues by the lectins resulted in reversion of the sialidase effect upon the endocytosis of *T. cruzi* by the macrophages in a dose-dependent way (particularly seen with WFA). The reversion of the sialidase effect is less intense with the lectins that block GalNAc than with those that block Gal. The exposure of subterminal Gal residues after desialylation is common on sialoglycolipids while the exposure of GalNAc is more typical for sialoglycoproteins (Suzuki, 1976). It was previously suggested that 63% of the sialic acid residues removed from the surface of bloodstream trypomastigotes of *T. cruzi* by the neuraminidase of *Clostridium perfringens* are associated with glycolipids, while only 37% are associated with glycoproteins (Souto-Padrón and De Souza, 1985). This may explain the higher intensity of reversion of the sialidase effect upon *T. cruzi*-macrophage interaction in our experiments, when PNA or RCA I was used. It is also interesting that DBA, a lectin which recognizes only terminal GalNAc (Nicolson, 1974), does not normally bind to *T. cruzi* trypomastigotes (Pereira et al., 1980), but after desialylation it reacts strongly with the parasites, reverting the sialidase effect on the parasite-macrophage interaction. Therefore, our results suggest that the enhanced uptake of bloodstream trypomastigotes of *T. cruzi* by macrophages after parasite desialylation is mediated by exposed residues of Gal and/or GalNAc.

Consistent with these results is the inhibitory effect of the simple addition of asialoerythrocytes or galactosyl-oligosaccharides to the interaction assay. The recognition of normal trypomastigotes is impaired when these Gal-terminal ligands were added during the *T. cruzi*-macrophage interaction.

The binding characteristics of the Gal/GalNAc-R of macrophages and hepatocytes are well characterized (reviewed in Reuter et al., 1982). It is a transmembrane glycoprotein that binds (at 4°C) and internalizes (above 10°C) glycoconjugates marked with residues of Gal or GalNAc and transports the ligands through endosomes to the lysosomal compartment. The receptor is

recycled back to the surface via the Golgi complex. Adhesion is Ca^{2+} -dependent, is prevented by 2 mM EDTA or EGTA (Kolb et al., 1979; Nagamura and Kolb, 1980) and is trypsin- and neuraminidase-sensitive (Ashwell and Morell, 1974). Similarly, *T. cruzi* interiorization by macrophages can be separated from the binding step by low temperature (Meirelles et al., 1982), and also the treatment of the macrophages with trypsin inhibits parasite uptake (Nogueira et al., 1980; Alcântara and Brener, 1980; Zenian and Kierzenbaum, 1983). Neuraminidase (Zenian and Kierzenbaum, 1983), cationized ferritin (Meirelles et al., 1984) and Limulin (Araújo-Jorge and De Souza, 1986) treatments of macrophages also inhibit interiorization of trypomastigotes by interfering in different ways with the macrophage surface sialic acid residues. *T. cruzi* recognition is also Ca^{2+} sensitive (unpublished results). Though indirect, these coincidences are also suggestive of the involvement of the macrophage Gal/GalNAc-R on the uptake of the trypomastigote stage of *T. cruzi*.

In conclusion, our present results – in parallel with those describing the interference of Gal and GalNAc on *T. cruzi*-macrophage interaction (Araújo-Jorge and De Souza, 1984) – strongly suggest that the lectin-like receptor for Gal/GalNAc located on the macrophage surface can mediate the interiorization of *T. cruzi*.

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