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Effect of carbohydrates, periodate and enzymes in the process of endocytosis of *Trypanosoma cruzi* by macrophages

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Summary

The effect of mild enzyme (trypsin, neuraminidase) treatment, periodate treatment and addition of carbohydrates (mono, di-, and polysaccharides) on the ingestion of *Trypanosoma cruzi* epimastigotes and trypomastigotes by mouse macrophages was studied. Trypsin treatment did not interfere with the ingestion of epimastigotes but did, however, increase the ingestion of trypomastigotes by mouse peritoneal macrophages. Neuraminidase and periodate treatment of the parasites increased the uptake of epi- and trypomastigote forms. The neuraminidase effect was partially blocked by galactose or N-acetylgalactosamine. Galactose, mannose, fucose, N-acetylglucosamine, and N-acetylgalactosamine had an influence on the ingestion of *T. cruzi* by macrophages. This effect was dependent on the strain of parasite tested, and the medium used to cultivate the epimastigotes. The results obtained, in conjunction with the work of others, suggest that glycoproteins and/or glycolipids on the parasite and/or macrophage surface are involved in the *T. cruzi*-macrophage interaction.

Key words: *Trypanosoma cruzi;* endocytosis; macrophage; cell surface; sialic acid.

Introduction

In the life of *Trypanosoma cruzi*, macrophages are probably the first cells in the vertebrate host to come in contact with the epi- and trypomastigote forms of the invertebrate host feces and urine. It has also been observed that certain strains of *T. cruzi*, when inoculated into mice, exhibit a tropism for macrophages by the bloodstream forms (Melo and Brener, 1978). In vitro studies have

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also shown that trypomastigotes of a strain with an in vivo tropism for macrophages are more easily ingested by mouse or chicken macrophages than trypomastigotes from myotropic strain of *T. cruzi* (Alcantara and Brener, 1978; Kipnis et al., 1979; Meirelles et al., 1980, 1982a).

Ultrastructural observations (Nogueira and Cohn, 1976; Maria et al., 1982) as well as studies with macrophages incubated at 4° C or preincubated with cytochalasin B (Meirelles et al., 1982b) showed that both epimastigote and trypomastigote forms of *T. cruzi* enter macrophages mainly by a process of endocytosis. In these studies, as well as in studies with non-professional phagocytic cells (Andrews and Colli, 1982) two steps in the process of infection of vertebrate cells by *T. cruzi* have been distinguished: (a) an attachment phase which can be observed in macrophages incubated at 4° C or in the presence of cytochalasin B, which is followed by (b) a phase of parasite interiorization which requires active participation of the macrophages.

In the cell-to-cell interaction processes the surface properties of the cells are important. Previous studies have shown that differences in surface properties exist between epimastigote and trypomastigote forms of *T. cruzi* as well as between different strains of the parasite (De Souza et al., 1977, 1978; Pereira et al., 1980; Nogueira et al., 1982, Araújo et al., 1980; Araújo and Remington, 1981). It has been shown that macrophages have membrane-associated macromolecules which specifically recognize the surface components of the particles with which they interact. Some of these components, such as the Fc and C3b receptors, play an important role in the process of phagocytosis (Huber et al., 1968; Lay and Nussenzweig, 1968). Other receptors recognize mannose, fucose and N-acetylglucosamine (Stahl and Gordon, 1982) or interact with lectins (Maddox et al., 1982; Goldman et al., 1976; Shepherd et al., 1981). Ultrastructural cytochemical studies have shown that *T. cruzi* has carbohydrate containing macromolecules on its surface (De Souza and Meyer, 1975; De Souza et al., 1978), as have studies utilizing agglutination with lectins with various specificities (Alves and Colli, 1974; Chiari et al., 1978; Pereira et al., 1980; Araújo et al., 1980) and biochemical studies (for a review see Colli et al., 1981).

In order to analyse the possible role of surface components of epimastigote and trypomastigote forms of *T. cruzi* during that interaction with macrophages, experiments were carried out in which (a) the surface on the parasite was altered by mild enzymatic and periodate treatment, and (b) some monosaccharides, disaccharides and polysaccharides were added to the medium just before the *T. cruzi*-macrophage interaction took place.

Materials and Methods

Parasites. Two strains of *Trypanosoma cruzi* were used. The Y strain, isolated from an acute human case of Chagas' disease (Silva and Nussensweig, 1953), which exhibits an in vivo tropism for murine macrophages and the CL strain, isolated from a triatomine bug, which has a tropism for muscle cells (Brener and Chiari, 1963; Melo and Brener, 1978).

For harvesting of bloodstream trypomastigotes (Y and CL strains) the blood from heavily infected Swiss Webster mice at the peak of the parasitemia (7–12th days) was collected by cardiac puncture with 3.8% sodium citrate as an anticoagulant. The blood was centrifuged at 150 *g* for 10 min, the pellet discarded and the supernatant recentrifuged at 900 *g* for 10 min. The pellet was then washed in Tyrode's solution at pH 7.2. Epimastigotes of the Y strain were cultivated either in Warren's (Warren, 1960) or LIT (Camargo, 1964) media for 3–5 days at 28° C. Epimastigotes of the CL strain (Clone CL14 isolated by Dr. E. Chiari) were cultivated for 48 h in LIT medium. After cultivation, the cells were collected by centrifugation and washed once with Tyrode's solution.

Macrophages. Cells were collected from the peritoneal cavities of uninfected Swiss mice after injection of 4 ml of Hank's balanced solution (HBS). Samples of a 0.5 ml suspension containing 2×10^6 mononuclear cells per ml were placed into Leighton tubes with flying cover-slips. After incubation for 40 min at 37° C the non-adherent cells were removed, the macrophage monolayers were washed twice with Tyrode's solution, 199 medium supplemented with 10% inactivated fetal calf serum was added and the cells were incubated for 24–48 h at 37° C.

Infection of the cells. Bloodstream trypomastigotes and epimastigotes were suspended in medium 199 in order to achieve a ratio of 10 parasites per macrophage when 0.5 ml of the suspension was added to the macrophage cultures. The number of macrophages in the preparation was estimated by counting 20 microscopic fields. Parasites were maintained in contact with the macrophages for a period of 1–2 h at 37° C, after which the cells were rinsed with Ringer's solution, fixed with Bouin's fixative and then stained with Giemsa. The incubation medium was composed of medium 199 without serum containing in some cases tested carbohydrates. The following carbohydrates were used at a concentration of 10 mg/ml, which was non toxic for the macrophages and the parasites: D(+)-galactose, D(+)-mannose, N-acetyl-D(+)-galactosamine, D(+)-xylose, L(−)-fucose, L(−)-arabinose, N-acetyl-D(+)-glucosamine, β-D(+)-allose, raffinose, mellibiose, β-lactose, α-lactose, yeast mannan, and a lipopeptidophosphoglycan (LPPG) isolated from epimastigotes of *T. cruzi*. This last carbohydrate was a gift from Drs. J. O. Previato and L. Mendonça-Previato (Institute of Microbiology, Rio de Janeiro, Brazil). The other carbohydrates were obtained from the Sigma Chemical Company and were of the highest grade of purity offered. In some experiments either the macrophage or the parasites were preincubated for 15 and 60 min, respectively, in the presence of some of the carbohydrates tested after which were allowed to interact maintaining or not the carbohydrate in the incubation medium.

Enzymatic treatment. Epimastigotes and trypomastigotes were washed with Tyrode's solution and incubated for 60 min at 37° C in the presence of 0.2 U/ml of neuraminidase (Sigma Chemical Company, type X, dissolved in Tyrode's solution, pH 6.2) or for 15 min at 37° C in the presence of 500 μg/ml of trypsin (Sigma, type III). Incubation was interrupted by washing the cells (in the case of neuraminidase treatment) or by addition of 30% serum to the incubation medium (in the case of trypsin treatment). In both cases the parasites were washed twice in Tyrode's solution after enzyme treatment.

Periodate treatment. Parasites were washed in Tyrode's solution and then incubated for 5 min at 37° C in the presence of 1 mM sodium periodate ($NaIO_4$) dissolved in PBS, pH 7.2.

Evaluation of results. After 1 or 2 h of macrophage-parasite interaction the cultures were fixed in Bouin's fixative and stained with Giemsa. The percentage of infected macrophages was determined by randomly examining at least 400 cells under high magnification with a Zeiss Universal Photomicroscope. The mean number of intracellular parasites per infected macrophages was also determined. The endocytic index was calculated by multiplying the percentage of infected macrophages and the mean number of parasites per infected cell. In each experiment the endocytic indexes obtained were normalized by considering the value obtained for controls as 100. The results obtained in experiments in which the parasites were treated with enzymes or periodate or when sugars were added to the incubation medium were expressed as a percentage in relation to controls. Experiments were repeated 2–7 times. Statistical analysis was performed using the F test to the log of the endocytic indexes obtained. Differences higher than 30% of controls were considered to be significant. The results are expressed as means ± standard deviations.

Results

Enzyme and periodate treatments of T. cruzi

Macrophages incorporated epimastigote and trypomastigote forms of *Trypanosoma cruzi* when incubated at 37° C for 1–2 h in a medium without serum. As was shown previously the endocytic index for epimastigotes is higher than that for bloodstream trypomastigotes (Meirelles et al., 1980, Table 1).

Pretreatment of *T. cruzi* epimastigotes with trypsin (500 μg/ml for 15 min at 37° C) did not influence their uptake by macrophages (Fig. 1). However, as was shown earlier (Nogueira et al., 1980; Kipnis et al., 1981) trypsin treatment of bloodstream trypomastigotes increased their uptake by macrophages about 7 times (Fig. 1). Trypsin treatment, under the conditions used here, did not interfere with parasite morphology, motility or viability.

Treatment of either epimastigote or trypomastigote forms of *T. cruzi* with neuraminidase increased the ingestion of the parasite by macrophages about 2 and 7 fold, respectively (Fig. 1). Pretreatment of epimastigotes or trypomastigotes with periodate doubled their uptake by macrophages (Fig. 1). The effect of neuraminidase was partially blocked by the addition of galactose and N-acetylgalactosamine in the interaction medium (Fig. 1). In order to find out if

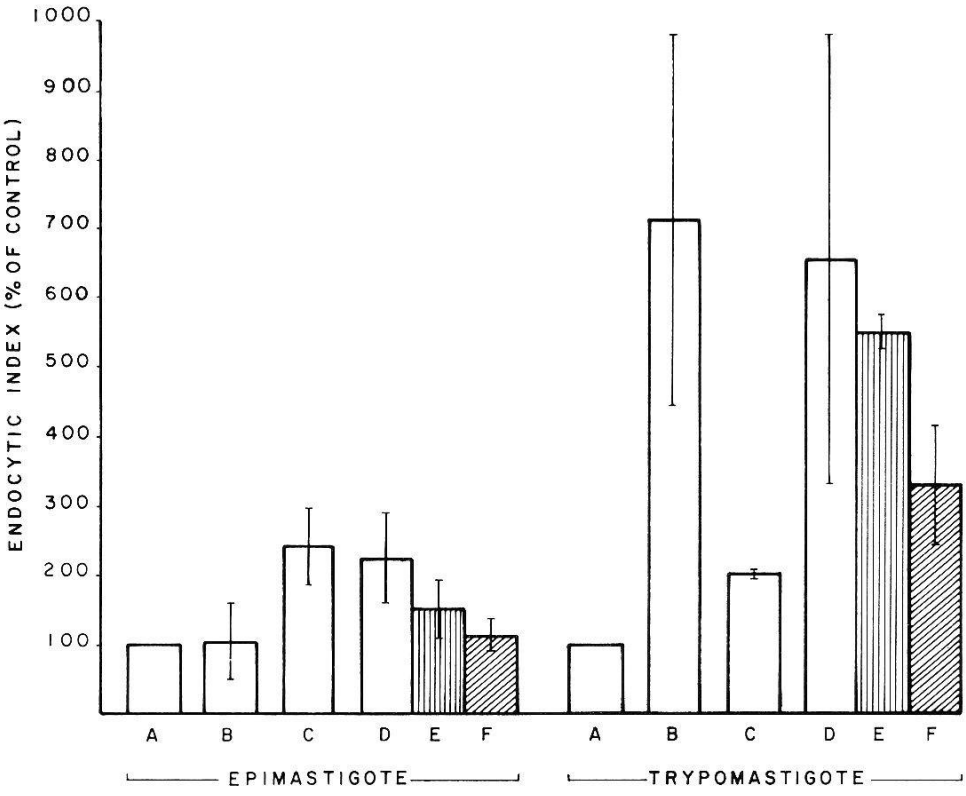


Fig. 1. Effect of pretreatment of epimastigote and trypomastigote forms of the Y strain of *T. cruzi* with trypsin (B), sodium periodate (C), or neuraminidase (D), on the interaction of the parasites for 2 h with macrophages. In some experiments using neuraminidase-treated parasites, galactose (E) or N-acetylgalactosamine (F) was added to the interaction medium. A = control. Means ± standard deviations of 2–7 experiments.

the neuraminidase used was active, its activity was determined by measuring the cellular electrophoretic mobility of the cells in a cytopherometer. We observed, as will be described in detail elsewhere, that neuraminidase treatment significantly reduced the negative charge of the parasite surface. The concentration of neuraminidase used was chosen based on previous observations (unpublished) which indicate that it had a maximum effect wihout interfering with parasite viability.

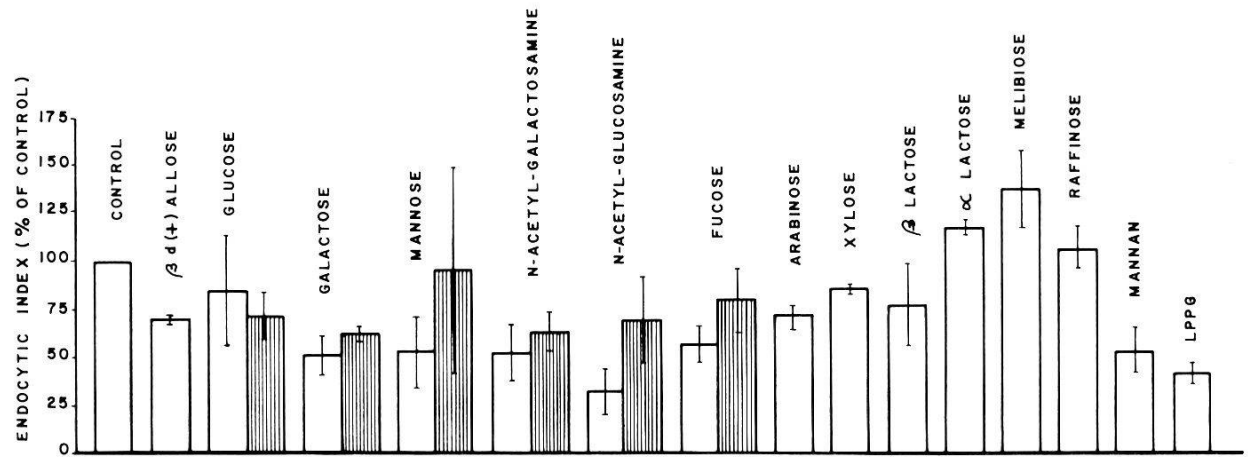
Effect of sugars on the uptake of T. cruzi by macrophages

Several monosaccharides, 3 disaccharides, 1 trisaccharide and 2 polysaccharides, one of which was isolated from epimastigotes of *T. cruzi*, were tested to determine their influence on the ingestion of *T. cruzi* by macrophages. We used a concentration of 10 mg/ml which, according to morphological criteria, did not alter the morphology of either the macrophages or the parasites nor changed the motility of the parasites.

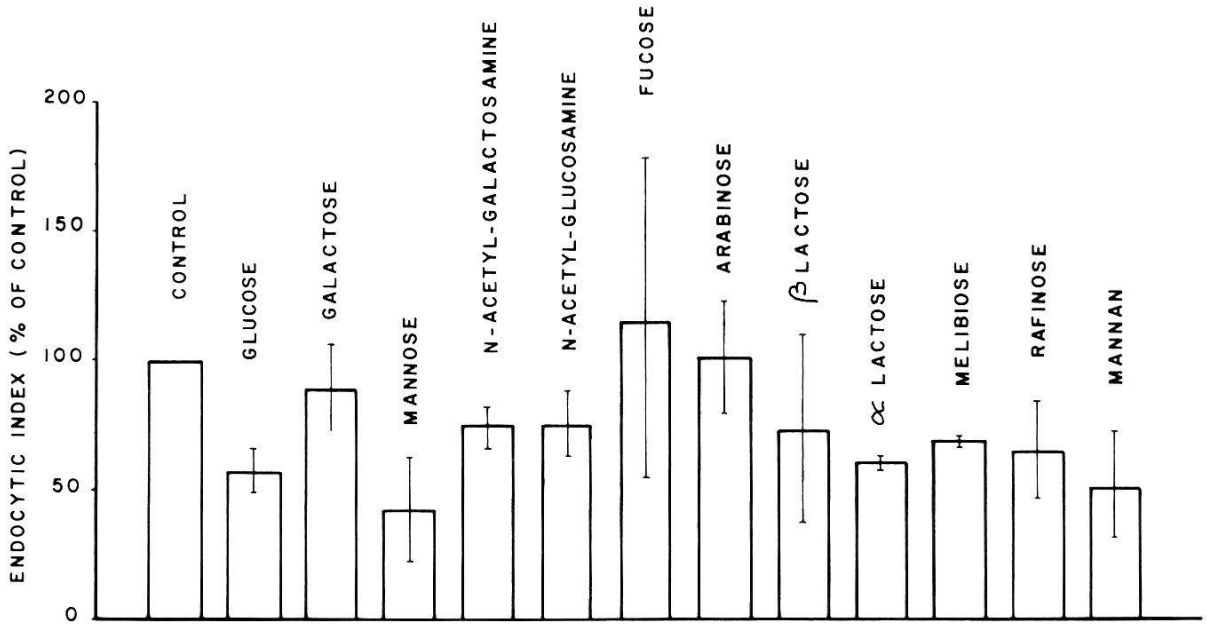
Some experiments were done using various concentrations of sugars. However, they confirm previous studies (Oda et al., 1982) which indicated that 10 mg/ml is an adequate concentration for this type of experiment. The effect of sugars on the ingestion of *T. cruzi* by macrophages was analysed only with a 1 h interaction time. Preliminary observations indicated that the effect of sugars diminished with the increase of the *T. cruzi*-macrophage interaction time. The effect of these carbohydrates on the ingestion by macrophages of epimastigotes of the Y strain of *T. cruzi* cultivated in the Warren's medium is summarized in Fig. 2. A significant inhibition of the endocytic index was observed with galactose, mannose, fucose, N-acetylgalactosamine and especially with N-acetylglucosamine, which showed an inhibition of about 70%. The two polysaccharides tested, mannan from Baker yeast and the LPPG isolated from *T. cruzi*, had a marked inhibitory effect. The other sugars tested did not have an effect on the ingestion of *T. cruzi* by macrophages although mellibiose had a slight stimulatory effect.

As is shown in Fig. 2, the inhibitory effect of some sugars on the uptake of epimastigotes of *T. cruzi* by macrophages was influenced by the medium in which the parasites were grown. The inhibitory effect was usually more pronounced when the parasites were cultivated in Warren's medium. These differences were most pronounced with mannose and N-acetylglucosamine. Only N-acetylgalactosamine significantly inhibited the uptake by macrophages of epimastigotes cultivated in the LIT medium.

It was also observed that the effect of some monosaccharides on epimastigote uptake by macrophages was dependent on the strain of *T. cruzi* used (Fig. 3). Glucose and mannose, which exerted a slight effect on the uptake of epimastigote of the Y strain cultivated in the LIT medium, had a marked effect on ingestion of CL strain epimastigotes grown in the same medium. The effect of N-acetylglucosamine, N-acetylgalactosamine, and galactose on the endocyt-


ENDOCYTIC INDEX (% OF CONTROL)
175
150
125
100
75
50
25
0
CONTROL
β d(+) ALLOSE
GLUCOSE
GALACTOSE
MANNOSE
N-ACETYL-GALACTOSAMINE
N-ACETYL-GLUCOSAMINE
FUCOSE
ARABINOSE
XYLOSE
β LACTOSE
α LACTOSE
MELIBIOSE
RAFFINOSE
MANNAN
LPPG

2


ENDOCYTIC INDEX (% OF CONTROL)
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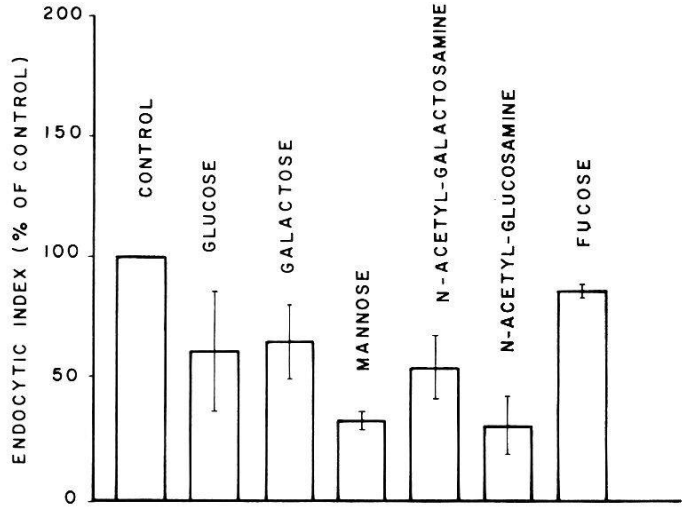
150
100
50
0
CONTROL
GLUCOSE
GALACTOSE
MANNOSE
N-ACETYL-GALACTOSAMINE
N-ACETYL-GLUCOSAMINE
FUCOSE
ARABINOSE
β LACTOSE
α LACTOSE
MELIBIOSE
RAFINOSE
MANNAN

3


ENDOCYTIC INDEX (% OF CONTROL)
200
150
100
50
0
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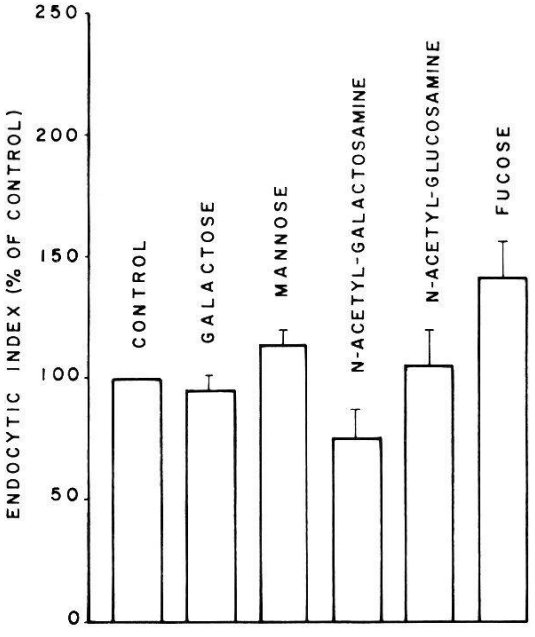
GLUCOSE
GALACTOSE
MANNOSE
N-ACETYL-GALACTOSAMINE
N-ACETYL-GLUCOSAMINE
FUCOSE

4


ENDOCYTIC INDEX (% OF CONTROL)
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200
150
100
50
0
CONTROL
GALACTOSE
MANNOSE
N-ACETYL-GALACTOSAMINE
N-ACETYL-GLUCOSAMINE
FUCOSE

Fig. 5

ic index, which was significant for parasites of the Y strain grown in the Warren's medium was not observed with the CL strain.

The effect of some sugars on the ingestion of Y and CL strain bloodstream trypomastigotes by macrophages was also determined. Glucose, galactose and N-acetylgalactosamine showed a slight inhibitory effect on the ingestion of trypomastigotes of the Y strain by macrophages. This effect was more pronounced with mannose and N-acetylglucosamine (Fig. 4). Galactose, mannose and N-acetylglucosamine did not interfere with the uptake of trypomastigotes of the CL strain by macrophages. A slight inhibitory effect was observed with N-acetylgalactosamine, whereas fucose significantly increased the ingestion of the parasites (Fig. 5).

Some experiments were done in which macrophages were incubated for 15 min in the presence of some sugars before interaction with Y strain epimastigotes and trypomastigotes. The sugars were maintained in this culture medium during *T. cruzi*-macrophage interaction. It was observed that only N-acetylglucosamine inhibited the ingestion of trypomastigotes by macrophages. This effect was abolished if the sugar was removed from the medium before macrophage-*T. cruzi* interaction. The other sugars either did not affect or potentiate the ingestion of epi- and trypomastigote forms by macrophages. Preincubation of Y strain epimastigotes (grown in Warren medium) for 1 h in the presence of various sugars followed by washing before interaction with macrophages did not interfere with their uptake by macrophages (not shown).

Discussion

Our studies confirm previous observations showing that treatment of bloodstream trypomastigotes of *T. cruzi* with trypsin led to a significant increase in the percentage of macrophages which incorporated parasites (Nogueira et al., 1980; Kipnis et al., 1981). It has been suggested that bloodstream trypomastigotes possess a trypsin-sensitive protein on their surface which has anti-phagocytic properties, inhibiting the ingestion of *T. cruzi* by macrophages. The protein has a molecular weight of 90,000 daltons and constitutes a major protein of the cell surface of bloodstream trypomastigotes of *T. cruzi* (Nogueira et al., 1981). Our results suggest that the trypsin-sensitive component is not present or

Fig. 2. Effect of the addition of sugars on macrophage ingestion of *T. cruzi* epimastigotes of the Y strain grown in Warren's (□) or in LIT (□) medium. LPPG = lipopeptidophosphoglucan isolated from epimastigotes of *T. cruzi*. Means ± standard deviations.

Fig. 3. Effect of the addition of sugars on macrophage ingestion of *T. cruzi* epimastigotes of the CL strain grown in LIT medium. Means ± standard deviations.

Fig. 4. Effect of the addition of sugars on the macrophages ingestion of *T. cruzi* bloodstream trypomastigotes of the Y strain. Means ± standard deviations.

Fig. 5. Effect of the addition of sugars on macrophage ingestion of *T. cruzi* bloodstream trypomastigotes of the CL strain. Means ± standard deviations.

exposed on the surface of epimastigotes since treatment of these forms with trypsin did not interfere with the uptake of epimastigotes by macrophages. These observations suggest that a new component appears or is exposed on the cell surface of *T. cruzi* during epimastigote-trypomastigote transformation rendering the parasites less susceptible to ingestion by macrophages.

These experiments show for the first time that treatment of either trypomastigotes or epimastigotes of *T. cruzi* with neuraminidase markedly influences the interaction of the parasite with macrophages thus suggesting that sialic acid localized on the surface of *T. cruzi* may be important in the parasite-macrophage interaction. The presence of sialic acid on the surface of *T. cruzi* has already been demonstrated by the binding of colloidal iron hydroxide particles at pH 1.8 (Martinez-Palomo et al., 1976; De Souza, 1978) and by the binding of lectins from wheat germ and *Limulus polyphemus* (Pereira et al., 1980) to the cell surface of *T. cruzi*. The increase in the uptake by macrophages of epimastigotes and trypomastigotes previously treated with periodate also suggests the presence of sialic acid on the surface of *T. cruzi* (Ogmundsdottir et al., 1978; Van Lenten and Ashwell, 1971). It is interesting to note that the neuraminidase treatment was about 4 times more effective in increasing the ingestion of trypomastigotes than of epimastigotes. This observation agrees with previous studies showing that bloodstream trypomastigotes of *T. cruzi* have a higher negative surface charge than epimastigotes (De Souza et al., 1977) and that sialic acid is the main component responsible for the negative surface charge of trypomastigotes (Souto-Padron et al., 1982). It is possible that *T. cruzi* cell surface sialic acid modulates the interaction process between parasite and macrophages in a manner similar to that described for erythrocytes and some glycoproteins (Czop et al., 1978; Jancik and Schauer, 1978; Ashwell and Morell, 1974; Kolb-Bachofen et al., 1982). In the case of *T. cruzi* we observed that the increased uptake of neuraminidase-treated parasites was partially blocked by galactose and by N-acetylgalactosamine. Further studies are necessary to determine the mechanism of sialic acid removal from the surface of *T. cruzi*. It is possible that a neuraminidase localized either on the parasite or the macrophage surface participate in this process. The presence of neuraminidase in *T. cruzi* was recently reported (Pereira, 1983).

Our results, as well as others recently reported, do not support the idea that the 90,000-dalton protein localized on the cell surface of bloodstream trypomastigotes of *T. cruzi* is an antiphagocytic factor (Nogueira et al., 1980, 1981) for the following reasons: (a) treatment of bloodstream trypomastigotes with trypsin or neuraminidase has basically the same effects, increasing the parasite's ingestion by macrophages, the susceptibility to complement-mediated lysis and diminishing the negative surface charge; (b) macrophages ingest 18 times more tissue cultured-derived trypomastigotes, which also have a 90,000-dalton protein localized on their cell surface (Nogueira et al., 1981) than bloodstream trypomastigotes (Meirelles et al., 1982a). The available data do not permit us to know

if trypsin and neuraminidase act on the same membrane-associated macromolecule. The observation that neuraminidase had an effect on epimastigote and trypomastigote forms whereas trypsin only interfered with trypomastigotes suggests that two molecules might be involved. Recently it was found that treatment of trypomastigotes with trypsin led to the disappearance of cell surface proteins and reduced their ability to infect epithelial cells (Andrews et al., 1982) whereas it enhanced the adhesion to and infectivity of the parasites for other cells (Henriquez et al., 1981).

Our results indicate that carbohydrates inhibit the interaction of *T. cruzi* with macrophages. They also show that this effect is not restricted to only one sugar and that it varies according to the form, the strain and the medium in which the parasite was cultivated. They also indicate that the nature of the medium in which Y strain epimastigotes are grown (Warren or LIT medium) may modify some properties of the parasite surface. Differences in the content of carbohydrates of epimastigotes grown in Warren or Chang medium have been reported recently (Gorin et al., 1981).

Our results show marked differences in the effect of some monosaccharides on macrophage ingestion of Y and CL strain epimastigotes cultivated in the same medium. Galactose, N-acetylgalactosamine and N-acetylglucosamine, which considerably inhibited the uptake of epimastigotes from the Y strain, did not interfere in the uptake of CL strain epimastigotes. The possibility that the differences found with various carbohydrates on the uptake of the parasites by macrophages may be related to the different tropism found in vitro and in vitro with the two strains of *T. cruzi* deserves future investigation.

We also observed an important inhibitory effect (about 50%) of a lipopeptidophosphoglucan (LPPG) on the uptake of epimastigotes of *T. cruzi* by macrophages. This result is of interest since LPPG is the main glycoconjugate isolated from *T. cruzi* and accounts for about 15% of the plasma membrane dry weight (Colli et al., 1981).

In the case of Y strain bloodstream trypomastigotes glucose, galactose, N-acetylgalactosamine, mannose and N-acetylglucosamine inhibited the uptake of the parasites by macrophages. The results obtained with bloodstream trypomastigotes from the CL strain are completely different. N-acetylglucosamine, which caused the most intense inhibition of the uptake of Y trypomastigotes does not interfere with the uptake of CL trypomastigotes. N-acetylgalactosamine slightly inhibited the ingestion of CL trypomastigotes by macrophages. The results obtained with the Y strain are in agreement with previous studies which showed N-acetylglucosamine to be the only monosaccharide which inhibited the infection of HeLa, LLC-MK2 (Andrews and Colli, 1981), bovine embryonic skin and muscle cells (Crane and Dvorak, 1982) by *T. cruzi* trypomastigotes. It is interesting to note that fucose which slightly inhibited the uptake of Y strain *T. cruzi* stimulated the uptake of epi- and trypomastigote forms of the CL strain by macrophages.

It is important to point out that the sugars which inhibited the ingestion of *T. cruzi* by macrophages are components of membrane-associated glycoproteins or polysaccharides of *T. cruzi* (Gonçalves and Yamaha, 1969; Gorin et al., 1981; Marcipar et al., 1982).

Previous studies have indicated that the interaction between *T. cruzi* and host cells involves the recognition of cell surface components of the parasite by cell surface components of the host cell or vice-versa and that these components may vary from epimastigote to trypomastigote forms (Alcantara and Brener, 1980; Henriquez et al., 1981; Zenian and Kierszenbaum, 1982; Meirelles et al., 1983). The data obtained by these authors associated with those reported here, indicate that cell surface carbohydrate-containing molecules are involved in the interaction of *T. cruzi* with vertebrate cells. Further studies are necessary to determine if cell surface lectin-like substances or glycosyl transferases, which have been suggested to play fundamental roles in the process of cellular recognition in eucaryotic cells (Rauvala et al., 1981; Olden et al., 1982), are involved in the process of *T. cruzi*-host cell interaction.

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