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Sialoglycoproteins and sialoglycolipids contribute to the negative surface charge of epimastigote and trypomastigote forms of *Trypanosoma cruzi*

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Epimastigote and trypomastigote forms of *Trypanosoma cruzi* have a net negative surface charge, as determined by direct measurement of the mean cellular electrophoretic mobility. Treatment of the parasites with neuraminidase reduces by 17 and 52% the mean electrophoretic mobility of epimastigote and bloodstream trypomastigote forms, respectively. Neuraminidase-treated cells recover their normal electrophoretic mobility if incubated for 2 h in the presence of fresh culture medium. The recovering process of epimastigotes is almost totally blocked by addition of inhibitors of either protein synthesis (puromycin) or *N*-glycosidically linked glycoprotein synthesis (tunicamycin). The recovering process of trypomastigotes is not totally inhibited by either puromycin or tunicamycin. Treatment of *T. cruzi* with trypsin reduces by 11 and 40% the mean electrophoretic mobility of epimastigote and bloodstream trypomastigote forms. Trypsin-treated cells recover their normal electrophoretic mobility if incubated for 4 h in fresh culture medium. The recovering process of trypomastigotes is partially inhibited by puromycin. The results obtained indicate that sialoglycoproteins and sialoglycolipids exist on the surface of *T. cruzi*, the latter being predominant on the surface of trypomastigotes.

Introduction

Evidence exists that *Trypanosoma cruzi* possesses a net negative surface charge which varies according to its evolutive stage [1–4]. Determination of the cellular electrophoetic mobility indicates that trypomastigotes have the higher, epimastigotes have the lower negative charge, and the charge of amastigotes lies in between [1,3–5]. Studies carried out in other cell types indicate that candidates most likely for anionic sites in the cell membrane are sulfate groups found in acid mucopolysaccharides, ionized phosphate groups found in phospholipids and charged carboxyl groups largely due to the presence of sialic acid [6–10].

Results obtained in the last years by ultrastructural cytochemistry [11–13], agglutination induced by lectins [14] and determination of the cellular electrophoretic mobility [3,4] suggest that sialic acid is present on the surface of *T. cruzi*. More recently, biochemical analysis confirmed these observations for epimastigotes [15,16].

In the present study, we examined the surface charge of epimastigote and trypomastigote forms of *T. cruzi* after treatment with trypsin or neuraminidase and incubation in fresh culture medium in the presence or absence of either puromycin, an inhibitor of protein synthesis or tunicamycin, an inhibitor of *N*-glycosylated glycoproteins. The results obtained indicate that cell surface sialic acid

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is associated with both glycoproteins and glycolipids.

Materials and Methods

Parasites. The Y and CL strains of *T. cruzi* were used. Bloodstream trypomastigotes have been maintained in Swiss albino mice by intraperitoneal inoculation. Trypomastigotes were collected on the 7th or 14th day of infection as previously described [3]. They were separated from blood cells by centrifugation in a gradient of metrizamide as described previously [17]. After isolation, bloodstream forms were washed with medium 199 without serum and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature. Tissue culture derived trypomastigotes were obtained from LA9 cells which were infected with bloodstream forms of *T. cruzi* [18]. Epimastigotes and trypomastigotes forms from axenic cultures were obtained according to the method of Chiari [19]. Clones of Y and CL strains, were cultivated in LIT medium [20] at 28°C for 2 days in order to obtain epimastigote forms. These forms were then transferred to a TSH-modified medium (M16) and after 4 days of culture provide $(7–8) \cdot 10^7$ parasites/ml. The advantage of M16 medium was the large rate of differentiation of epimastigotes into trypomastigotes yielding up to 90–95% trypomastigotes. Trypomastigote forms were obtained from M16 medium after 168 h of cultivation. All cells were washed in 0.1 M phosphate buffer and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature.

Enzymatic treatment. Axenic cultured epimastigotes and bloodstream trypomastigotes were collected by centrifugation, washed twice in phosphate-buffered saline, resuspended in phosphate-buffered saline ($1 \cdot 10^6$ cells/ml) and then incubated for 30 min at 37°C in the presence of 0.25 U/ml neuraminidase (Sigma type X) in Tyrode's solution (pH 6.0) or for 5 min at 37°C in the presence of 500 μg/ml trypsin (Sigma type III) in Ringer's solution (pH 7.2). Trypsin was inhibited by the addition of bovine serum (final concentration, 10%) to the test tube. After enzyme treatment, the cells were collected by centrifugation, washed twice in phosphate-buffered saline and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. In some experiments, enzyme-treated parasites were reincubated in fresh culture medium and samples were collected after 1, 2 and 4 h, washed with phosphate-buffered saline and fixed in glutaraldehyde.

Microelectrophoretic measurement. The electrophoretic mobility of the cells was determined in a Zeiss cytopherometer with a current of 4–6 mA and a final voltage of 100 V. The cell suspension was placed into the chamber and then allowed to equilibrate for 10 min. Measurements were made at a temperature of 25°C in a 0.85% sodium chloride solution with an ionic strength of 0.145 $\text{mol} \cdot \text{dm}^{-3}$, at pH 7.2. We also determined the electrophoretic mobility of living parasites. In this case, measurements were made at 4°C to prevent the movement of the cells. When current was switched on, we measured the time necessary for one cell to travel across two vertical lines, separated by a distance of 16 μm. Then, the polarity was reversed and the time was measured again for the cell travelling in the opposite direction. 50–100 cells were measured for each sample analysed.

Calibration of the equipment was made by measuring the electrophoretic mobility of fresh human erythrocytes. Statistical analysis was performed using the *t*-test.

Effect of puromycin and tunicamycin. Enzyme-treated parasites were washed once with phosphate-buffered saline and reincubated in fresh culture medium (Warren's medium [21]) for epimastigotes; M16 medium for axenic culture-derived trypomastigotes; 199 medium + 10% fetal bovine serum for bloodstream trypomastigote) and samples were collected after 1, 2 or 4, washed with phosphate-buffered saline and fixed in glutaraldehyde. In some experiments, the cells were submitted to three cycles of trypsin treatment of trypomastigotes, followed by incubation for 4 h in the presence of culture medium containing puromycin, before glutaraldehyde fixation. In some tubes, 10 μg/ml puromycin or 1 μg/ml tunicamycin was added to the culture medium; these concentrations were selected based on previous observation that 10 μg/ml puromycin blocks *T. cruzi* protein synthesis, and that 1 μg/ml tunicamycin inhibits parasite growth by about 90%. In order to determine if these antibiotics interfered with parasite viability, epimastigotes were in-

cubated for 4 h in the presence of 10 μg/ml puromycin or 1 μg/ml tunicamycin after which were washed twice with fresh medium and incubated at 28°C in the presence of fresh culture medium. At intervals of 24 h, samples were collected and the number of cells/ml was determined in a Neubauer chamber.

Results

Puromycin or tunicamycin, when added to the culture medium, inhibited the process of division of epimastigotes. In the case of puromycin, we only used a concentration of 10 μg/ml, which has been shown to inhibit protein synthesis of *T. cruzi*. In the case of tunicamycin, various concentrations were tested. We observed that even at low concentration, such as 1 ng/ml, tunicamycin partially inhibited the growth of epimastigotes of *T. cruzi*. At a concentration of 1 μg/ml, an inhibition of growth of about 90% was observed and this concentration was selected for further studies.

Epimastigotes of *T. cruzi* incubated for 4 h in the presence of 10 μg/ml puromycin or 1 μg/ml tunicamycin and then washed and incubated in fresh culture medium had their growth inhibited in the first day of cultivation. However, after 48 h they start to divide so that after 96 h the cultures reach the stationary phase of growth (Table I).

As previously shown [3], epimastigote and trypomastigote forms of *T. cruzi*, when suspended in a saline solution with an ionic strength of 0.145 $mol \cdot dm^{-3}$ at pH 7.2, have a characteristic mean electrophoretic mobility. Glutaraldehyde fixation did not influence the electrophoretic mobility of the cells since the same values were obtained after fixation as with living cells maintained at 4°C. Table II shows the mean electrophoretic mobility of epimastigotes incubated in culture medium for 0.5–3 h in the presence of various concentrations of tunicamycin. Only after 2 h of incubation a decrease in the mean electrophoretic mobility of epimastigotes was observed. The mean electrophoretic mobility of control, as well as of bloodstream trypomastigotes incubated for 3 h in the presence of 1 μg/ml tunicamycin, is also shown. A decrease in about 30% of the mean electrophoretic mobility of the parasites was observed.

Treatment with trypsin reduced by 11 and 40% the mean electrophoretic mobility of epimastigote and trypomastigote forms, respectively. In the case of trypomastigotes, similar results were obtained with parasites obtained from the bloodstream of

TABLE I

RECOVERY OF GROWTH OF EPIMASTIGOTES PREVIOUSLY INCUBATED IN THE PRESENCE OF PUROMYCIN OR TUNICAMYCIN

The inoculum was of $5.0 \cdot 10^6$ cells/ml.

Experimental condition	Number of cells/ml of culture after (h)			
	24	48	72	96
Control	$1 \cdot 10^7$	$2.3 \cdot 10^7$	$2.3 \cdot 10^7$	$2.5 \cdot 10^7$
Puromycin (10 μg/ml)	$4.4 \cdot 10^6$	$5.7 \cdot 10^6$	$1.5 \cdot 10^7$	$1.8 \cdot 10^7$
Tunicamycin (1 μg/ml)	$5.6 \cdot 10^6$	$9.4 \cdot 10^6$	$1.7 \cdot 10^7$	$2.2 \cdot 10^7$

TABLE II

EFFECT OF TUNICAMYCIN ON THE MEAN ELECTROPHORETIC MOBILITY (EPM) OF EPIMASTIGOTE AND TRYPOMASTIGOTE FORMS OF *T. CRUZI*

Data are from 100 cells. In all instances, $I = 0.145$ at pH 7.2.

Developmental stage	Concn. (μg/ml)	Mean EPM ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) ± S.D.		
		30 min	2 h	3 h
Epimastigote	none	−0.63 ± 0.03	−0.64 ± 0.04	−0.64 ± 0.04
	0.1	−0.63 ± 0.03	−0.61 ± 0.04	−0.54 ± 0.05
	0.5	−0.63 ± 0.03	−0.62 ± 0.05	−0.54 ± 0.04
	1.0	−0.64 ± 0.03	−0.60 ± 0.04	−0.54 ± 0.04
Trypomastigote	none	–	–	−1.15 ± 0.17
	1.0	–	–	−0.81 ± 0.04

TABLE III

EFFECT OF PUROMYCIN AND TUNICAMYCIN ON THE RECOVERY OF THE CELLULAR ELECTROPHORETIC MOBILITY (EPM) OF NEURAMINIDASE-TREATED AND TRYPSIN-TREATED EPIMASTIGOTES AND TRYPOMASTIGOTES OF *T. CRUZI*

n.d., not determined.

Developmental stage	Enzymatic treatment	Mean EPM ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$)								
		Control	Enzyme treated	Recovery						
				normal medium			normal medium + puromycin			normal medium + tunicamycin (4h)
				1 h	2 h	4 h	1 h	2 h	4 h	
Epimastigote	trypsin	-0.63 ± 0.03	-0.52 ± 0.02	-0.59 ± 0.03	-0.61 ± 0.05	-0.66 ± 0.08	-0.51 ± 0.05	-0.49 ± 0.03	-0.47 ± 0.05	-0.46 ± 0.03
Trypomastigote	trypsin	-1.01 ± 0.04	-0.65 ± 0.03	n.d.	-0.77 ± 0.05	-1.02 ± 0.05	n.d.	n.d.	-0.86 ± 0.06	-0.85 ± 0.03
Epimastigote	neuraminidase	-0.63 ± 0.03	-0.50 ± 0.03	-0.60 ± 0.03	-0.64 ± 0.04	-0.63 ± 0.03	-0.51 ± 0.04	-0.44 ± 0.04	-0.46 ± 0.04	-0.43 ± 0.02
Trypomastigote	neuraminidase	-1.01 ± 0.04	-0.57 ± 0.04	n.d.	-1.04 ± 0.14	n.d.	n.d.	-0.84 ± 0.07	n.d.	-0.86 ± 0.04

infected mice, from the supernatant of cell cultures or from axenic cultures. Control epimastigotes had a mean electrophoretic mobility of -0.65, while trypsin-treated cells had a mean electrophoretic mobility of $-0.58\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$. Control trypomastigotes had a mean electrophoretic mobility of -1.01 and it was reduced, by trypsin treatment, to $-0.65\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$ (Table III).

Trypsin-treated parasites recovered their normal mean electrophoretic mobility if, after enzyme treatment, they were incubated in a fresh culture medium. Complete recovery of the surface charge was observed after an incubation time of 2 and 4 h for epimastigote and trypomastigote forms, respectively. If the incubation time was shorter, only partial recovering of the surface charge was observed (Table III).

Recovering of the electrophoretic mobility of trypsin-treated epimastigotes was totally inhibited by addition of puromycin or tunicamycin to the culture medium. In addition, both substances induced a decrease in the mean electrophoretic mobility of epimastigotes, reaching values lower than that obtained with enzymatic treatment (Table III). In contrast, the recovering of the electrophoretic mobility of trypsin-treated trypomastigotes was only partially inhibited by addition of puromycin or tunicamycin (Table III). The grade of inhibition was basically the same if the trypomastigotes were submitted to three cycles of the trypsin treatment followed by incubation for 4 h in fresh culture medium containing puromycin.

Treatment with neuraminidase reduced by 17 and 52% the mean electrophoretic mobility of epimastigote and bloodstream trypomastigote forms, respectively. All trypomastigotes, obtained from three different sources, showed the same susceptibility to the neuraminase treatment. Neuraminidase-treated parasites recovered their normal surface charge when incubated for 2 h in fresh culture medium (Table III). The recovering process of epimastigotes was totally blocked by either 10 μg/ml puromycin or 1 μg/ml tunicamycin. As observed with trypsin-treated cells, incubation of neuraminidase-treated epimastigote in the presence of puromycin or tunicamycin induced a decrease in their mean electrophoretic mobility (Table III). In the case of trypomastigotes, puromycin and tunicamycin blocked only partially (by 37 and 32%, respectively) the recovering of the electrophoretic mobility. The inhibitory effect, although observed in all experiments, was less pronounced with puromycin than with tunicamycin (Table III).

Discussion

The following data indicate that sialic acid is present on the surface of *T. cruzi*: (a) Colloidal iron hydroxyde particles at pH 1.8 bind to the surface of the parasite. The binding is abolished if the parasites are incubated in the presence of neuraminidase before incubation [11–13]. (b) The agglutination of epimastigotes by the wheat germ lectin is abolished by previous treatment of the parasites with neuraminidase or by addition of *N*-acetylneuraminic acid to the incubation medium. In addition, the lectin from *Limulus polyphemus*, which is specific for sialic acid, agglutinates epimastigotes. Trypomastigotes are less sensitive to this lectin and amastigotes do not agglutinate [14]. (c) All developmental stages of the *T. cruzi* life cycle have a net negative surface charge as determined by cellular electrophoresis [1–4]. The surface charge, however, is reduced if the parasites are treated with neuraminidase [3,4]. (d) Treatment of the bloodstream trypomastigotes with neuraminidase render them able to activate the alternative complement pathway [22] and to be easily ingested by macrophages [23]. (e) By thin-layer and gas-liquid chromatography it was shown recently that epimastigotes contain *N*-acetyl- and *N*-glycolylneuraminic acids [15]. Sialic acid was also seen forming glycolipids [16].

We observed that treatment of *T. cruzi* with neuraminidase reduces the electrophoretic mobility of epimastigote and trypomastigote forms in about 17 and 52%, respectively. Therefore, after neuraminidase treatment epi- and trypomastigote forms have basically the same surface charge. This observation suggests that trypomastigotes have much more exposed cell surface sialic acid residues sensitive to the neuraminidase of *Clostridium perfringens* than epimastigotes. The sialic acid-containing sites reappear on the cell surface when neuraminidase-treated cells are incubated for 2 h in fresh culture medium. This observation favors the idea that the surface charge is not given by components of the medium which are adsorbed to

the parasite's surface. Based on the fact no radioactivity was found in the sialic acids of cells incubated with either [^{3}H]acetate or *N*-acetyl[^{3}H]mannosamine, it has been suggested that epimastigotes of *T. cruzi* are unable to synthesize sialic acid from its precursors [15].

It is well known that sialic acid can be associated either to glycoproteins or glycolipids or to both [24]. In the case of glycoproteins, it can be associated to either *N*- or *O*-glycosidically-linked oligosaccharide chains. Previous studies have shown that puromycin, an inhibitor of protein synthesis, blocks completely the synthesis of both groups of glycoproteins. Tunicamycin, a nucleoside antibiotic produced by *Streptomyces lysosuperificus*, inhibits the assembly of the oligosaccharide core in glycoprotein biosynthesis at the initial dolichol-mediated step by acting as inhibitor of the *N*-acetylglucosamine-1-phosphate transferase [25,26]. The biosynthesis of *O*-linked glycoproteins is not affected by tunicamycin. Our data show that puromycin and tunicamycin abolish completely the recovery of the surface charge when neuraminidase-treated epimastigotes are incubated in a fresh culture medium. This observation suggests that most of the sialic acid-containing molecules located on the surface of epimastigotes, and sensitive to the neuraminidase which was used by us, are *N*-linked glycoproteins. It is also in agreement with the results obtained showing that incubation of epimastigotes in the presence of tunicamycin led to a reduction of the electrophoretic mobility of about 16%, about the same value obtained when the parasites are incubated in the presence of neuraminidase. In addition, we observed that when enzyme-treated epimastigotes are incubated for 4 h in the presence of puromycin or tunicamycin there was a decrease of the mean electrophoretic mobility which reached values smaller than those obtained with the enzymatic treatment. This observation suggests that *N*-linked glycoproteins play an important role in maintainence of the surface charge of epimastigotes. It has been shown that sialic acid associated to glycolipids exists in epimastigotes of *T. cruzi* [16]. We cannot exclude the possibility that the glycolipid-associated sialic acid is not well exposed on the surface of living cell and therefore it is not accessible to the neuraminidase treatment. Previous studies using the isolated glycolipid show that it is sensitive to the neuraminidase from *C. perfringens* [16].

In the case of trypomastigotes of *T. cruzi*, we observed that both puromycin and tunicamycin only partially blocked the recovery of the normal surface charge when the enzyme-treated parasites were incubated in a fresh culture medium. Puromycin blocked, slightly, although consistently, more than tunicamycin. The results obtained, which are summarized in Table IV, suggest that 43% of the net surface charge of trypomastigotes is given by sialic acid. Most of the sialic acid (27% of the charge or 63% of the total sialic acid) would be asociated with glycolipids. About 16% of the net charge or 37% of the sialic acid would be given by sialic acid associated to glycoproteins, mainly (32%) of the *N*-linked type. This suggestion is supported by the observation that a reduction of

TABLE IV

CONTRIBUTION OF SIALIC ACID ASSOCIATED TO GLYCOPROTEINS OR GLYCOLIPIDS TO THE SURFACE CHARGE OF TRYPOMASTIGOTES

Values obtained based on data shown in Table III. EPM, electrophoretic mobility.

Nature of the glycoconjugate	Type	% of the EPM	% Relative to all sialic acid detected
Glycoprotein	tunicamycin-sensitive (*N*-linked)	14	32
	tunicamycin-insensitive (*O*-linked)	2	5
Glycolipid	total	16	37
		27	63

about 30% of the electrophoretic mobility of trypomastigotes occurs when they are incubated for 3 h in the presence of tunicamycin. It is important to point out, however, that these considerations are valid only to sialic acid-containing molecules accessible and sensitive to the neuraminidase used.Trypsin treatment reduced considerably, although less than neuraminidase, the surface charge of trypomastigotes. The cells recover the normal surface charge when incubated in fresh culture medium. However, the recovery time is longer (4 h) than in the case of neuraminidase treatment (2 h). It is possible that once modified by enzymatic treatment the membrane components are internalized and recycled, as has been shown to occur in other eukaryotic cells [27].

We observed that puromycin did not block completely the recovery of the surface charge of trypsin-treated trypomastigotes. This observation suggests that part of the cell surface proteins were already synthesized and are part of the cytoplasmic pool. Therefore, they are not affected by puromycin. The same occurs with tunicamycin. Previous studies have shown that treatment of trypomastigotes with trypsin removes a glycoprotein, with a molecular weight of 90000 [28,29] or 80000 [30]. This treatment (a) increases the uptake of bloodstream trypomastigotes by macrophages [22,23,28], (b) renders trypomastigotes able to activate the alternative complement pathway [22] and (c) decreases the ability of trypomastigotes to infect vertebrate cells [28]. It is possible that the reduction in the surface charge by treatment of trypomastigotes with trypsin is responsible for all these effects since they are also observed when the parasites are treated with neuraminidase. Further studies are necessary to determine if in *T. cruzi* the effects observed when the parasites are treated with trypsin or neuraminidase result from interference with the same membrane-associated macromolecule.

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