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Mechanism of cell invasion by *Trypanosoma cruzi:* importance of sialidase activity

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Summary

The sialidase activity of trypomastigotes of *Trypanosoma cruzi* and its relationship to the ability of different stocks of the organism to infect cultured cells was examined. Sialidase activity in lysates of trypomastigotes was confirmed and shown to be present in organisms of four different stocks of *T. cruzi*. In addition, sialidase activity was detected in sera of mice acutely infected with organisms of each of the stocks of *T. cruzi* examined. Erythrocytes from these mice were agglutinated by peanut lectin, suggesting sialidase activity in vivo. Treatment of normal mouse peritoneal macrophages with sera from acutely infected mice resulted in an increased capacity of the cells to internalize blood trypomastigotes. IgM or IgG antibodies specific to *T. cruzi* were not detected in the sera displaying sialidase activity. Treatment of parasites and/or normal mouse macrophages with *Vibrio cholerae* neuraminidase, however, had little effect in the rate of internalization of parasites. Treatment of L 929 mouse fibroblasts with neuraminidase reduced significantly the rate of infection of the cells with blood trypomastigotes. Anti-sialidase activity developed and was detected in sera of infected mice and humans, suggesting that the neuraminidase activity of the parasite may play a significant role in the invasion of host cells only during the initial phase of the infection.

Key words: sialidase; *Trypanosoma cruzi;* neuraminidase; macrophages.

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Introduction

Trypanosoma cruzi, the causative agent of Chagas' disease, circulates in the bloodstream of the mammalian hosts as non-replicating trypomastigotes which are able to invade almost any type of cell (Brener, 1980). The invasive step is crucial for the life cycle of the parasite since it has to become intracellular to multiply. Different stocks of *T. cruzi,* however, have different capacities to invade mammalian cells and a clear explanation for this observation has not been provided yet (Brener, 1980; Doyle et al., 1984). The difference in invasiveness may be related to the capacity of parasites of different stocks to produce or to activate factors which facilitate interiorization. In this context, recent reports (Pereira, 1983a; Libby et al., 1986) have suggested that the high capacity of trypomastigotes of certain stocks of *T. cruzi* to invade mammalian myocardial and endothelial cells is related to the ability of the organisms to chemically modify the cell membrane surface by desialization.

Because of reports showing neuraminidase activity in supernatants of cultured trypanosomes (Pereira, 1983b; Libby et al., 1986) it was considered of interest to determine neuraminidase activity in the serum of mice acutely infected with *T. cruzi* and whether this activity would influence interiorization of the parasites within phagocytic and non-phagocytic cells.

Materials and Methods

Reagents. Lectin from *Arachis hypogaea* (Peanut agglutinin, PNA), bovine serum albumin (BSA) and ethyleneglycol were purchased from Sigma Chemicals Co. (St. Louis, MO). Neuraminidase from *Vibrio cholerae* (E.C.3.2.1.18, specific activity >20 units/mg protein, VCNase) was from Calbiochem-Behring (La Jolla, CA). Fetal calf serum (FCS), Hanks' balanced salt solution (HBSS), Eagle's minimal essential medium (MEM) and RPMI-1640 tissue culture medium were obtained from GIBCO Laboratories (Grand Island, N.A.).

Mice. Swiss-Webster (SW) males, 4-week-old, 20–22 g (Simonsen Laboratories, Gilroy, CA) were used to obtain bloodstream forms of *T. cruzi.* Mice 8–10 weeks old were used to obtain peritoneal macrophages.

Trypanosoma cruzi. The Y, Tulahuén, CL and MR stocks were used because of their differences in cell and tissue tropism (Taliaferro and Pizzi, 1955; Melo and Brener, 1978; Brener, 1980). All stocks are maintained in our laboratory by periodic transfers in adult, male, SW mice. Blood from acutely infected mice was collected 4 or 7 days after infection with the Y or Tulahuén stocks and 7 to 10 days after infection with the Cl or MR stocks. Heparinized blood was diluted 1 : 1 with 6% dextran in HBSS, incubated 45 min at room temperature and centrifuged at 60 *g* for 15 min at room temperature. Trypomastigotes in the supernatant were collected and centrifuged again at 600 *g* for 20 min. The pelleted organisms were resuspended in HBSS and washed once by centrifugation as above. Final suspensions were made in RPMI 1640 supplemented with 5% heat-inactivated (60° C) FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) or in MEM supplemented with antibiotics.

Homogenates of blood trypomastigotes were prepared by three cycles of freezing and thawing 4×10^7 organisms in 1 ml of sterile distilled water. The lysate was centrifuged at 400 *g* for 15 min in the cold (4° C) and the supernatant used immediately. As control, VCNase (5 mU/ml in HBSS) was processed in parallel with the *T. cruzi* lysate.

Infection of cell monolayers. Monolayers of normal mouse peritoneal macrophages (MØ) were established in either four or eight-chamber tissue culture slides (Lab-Tek Products, Napperville, Ill.) as previously described (McCabe et al., 1985). Monolayers of L 929 cells (ATCC CCLI), a subcutaneous mouse fibroblast cell line, were also used. Trypomastigotes were added to the monolayers at a parasite/cell ratio of 3:1 or 5:1 and the cells incubated for 4 or 20 h at 37°C in an 5% CO_2 incubator. At the end of each experiment, the monolayers were exhaustively rinsed with HBSS, fixed with absolute methanol and stained with Giemsa stain. The percent infected cells was determined by random examination of a minimum of 300 cells by microscopy under high magnification. All experiments were run at least 3 times with identical design and each experimental point was set up in duplicate.

The effect of exogenous neuraminidase on the interiorization process of *T. cruzi* was examined by treating trypomastigotes only, trypomastigotes and MØ or L 929 cells, or cells only with VCNase for 30 min at 37°C before infection or by adding parasites and VCNase to the cell monolayers and overnight incubation.

The effect of serum factors on the interiorization of *T. cruzi* was examined by infecting MØ or L 929 cells in the presence of 10 to 50% of serum from mice infected with 1×10^6 trypomastigotes 4 to 7 days prior to the experiment. Sera for this experiment were centrifuged at $5{,}000\times g$, filtered through a 0.45 μm filter (Millex-HA, Millipore Corp., Bedford, MA) and used immediately. Specific IgM and IgG antibodies to *T. cruzi* in these sera were examined by using an enzyme-linked immunosorbent assay (Araujo and Guptill, 1984). Control sera from normal mice were processed similarly.

Treatment of fixed cell monolayers with neuraminidase. Monolayers of L 929 cells and of peritoneal macrophages from normal mice or mice that had been injected with *Corynebacterium parvum* were cultured in plastic dishes for 24 h, washed with HBSS, air dried and fixed with 1% para-formaldehyde in HBSS for 30 min at room temperature (Rosen et al., 1985). Each duplicate monolayer was treated with 15 mU of VCNase in acetate buffer (100 mM Na Cl, 50 mM sodium acetate, 4 mM calcium chloride, pH 5.5) for 30 min at 37°C. Control monolayers were treated with buffer only. Adherence of *T. cruzi* trypomastigotes to VCNase-treated or control monolayers was assayed by overlaying the cells with 2×10^6 blood trypomastigotes of the Y stock of *T. cruzi* suspended in 150 μl of MEM for 30 min at 37°C for MØ or 90 min for L 929. Thereafter, the monolayers were washed and stained as described above.

Assay for sialidase activity. The assay is based on the ability of PNA to agglutinate mature red blood cells (RBC) after their desialization (Lotan et al., 1975). The technique described by Pereira (1983b) was used. Briefly, 2% suspensions of test or control RBC were mixed with serial two-fold dilutions of PNA (starting concentration was 0.5 mg/ml). After incubation for 2 h at room temperature, the plates were read visually and the highest dilution showing a well defined hemagglutination pattern was considered the agglutinating titer. All assays were performed in U-bottom tissue culture plates (Costar, Cambridge, MA). The diluent was 0.01 M sodium phosphate buffered saline, pH 6.5, containing 2% BSA and 30% ethyleneglycol.

Sialidase activity of T. cruzi was investigated by using RBC from normal mice preincubated for 4 h at 37°C with either live bloodstream parasites or homogenates prepared with them. After incubation with live or homogenized parasites, the RBC were pelleted and washed four times with PBS containing 2% BSA.

Sialidase activity during experimental infection with *T. cruzi* was investigated by using RBC from acutely infected mice or RBC from normal mice. The RBC from normal mice were preincubated for 2 h with sera from mice that had been infected 2 to 14 days before with 7×10^4 bloodstream forms of *T. cruzi*. After incubation with sera, the RBC were washed four times with PBS containing 2% BSA and used in the assay for sialidase activity.

The development of anti-sialidase activity in mice or humans infected with *T. cruzi* was determined by examining the ativity of VCNase on normal human RBC incubated in the presence of sera from acutely and chronically infected humans and mice.

Table 1. Effect of exogenous neuraminidase on the infection of mammalian cells by *T. cruzi*[a]

Treatment	Conc. of VCNase (mU/ml)	% of infected cells[b]	
		L 929 cells	macrophages
Parasites	None	19.2 ± 1.2	30.1 ± 1.6
	2	16.7 ± 3.2 (−13)	34.6 ± 3.3 (+15)
	20	not done	36.0 ± 3.7 (+20)
	200	20.0 ± 1.5 (+1)	38.0 ± 5.6 (+26)
Host cells	None	20.1 ± 1.8	31.6 ± 0.8
	2	10.0 ± 2.0 (−50)**	40.1 ± 4.0 (+27)
	20	15.0 ± 0.8 (−25*)	32.5 ± 5.7 (+3)
	200	18.2 ± 0.8 (−5)	22.9 ± 2.1 (−28)**
Host cells and parasites	None	23.5 ± 3.5	29.0 ± 0.7
	2	12.0 ± 2.0 (−49)**	35.3 ± 3.7 (+22)
	20	not done	38.9 ± 5.9 (+34)*
	200	11.8 ± 0.2 (−50)**	29.4 ± 5.4 (+1)

[a] Parasites or host cells were incubated with VCNase for 30 min at 37° C before infection; or parasites and VCNase were added at the same time to the monolayers.

[b] The results are representative of four experiments and are presented as the mean ± S. D. of cells infected for 20 d. The percent increase (+) or decrease (−) with respect to the corresponding control is given in parenthesis. The % of infected cells were compared against their corresponding controls and differences statistically significant are presented as ** ($p < 0.001$) or * ($p < 0.05$) (Student's t test).

Results

Effect of neuraminidase on infection of cells by T. cruzi. The results with trypomastigotes of the Y stock are shown in Table 1. Similar results were noted in experiments performed with organisms of the CL stock (data not shown). Pretreatment of parasites for 30 to 150 min with up to 200 mU/ml of VCNase did not result in statistically significant differences in the proportion of infected MØ or L-cells after 4 or 20 h of incubation.

Pretreatment of MØ with 200 mU/ml of VCNase and of L 929 cells with 2 or 20 mU/ml for 30 min followed by incubation of the cells with trypomastigotes for 20 h resulted in a statistically significant decrease of the percent infected cells. In both cases no effects were seen after 4 h of infection of the cells. The presence of 20 mU/ml of VCNase during the entire incubation period increased the uptake of parasites by MØ ($p < 0.05$). In contrast the rate of infection of L 929 cells was significantly ($p < 0.001$) reduced at every concentration of VCNase assayed (Table 1).

Adherence of T. cruzi to fixed cells treated with VCNase. Treatment of fixed cells with 15 mU of VCNase significantly ($p < 0.02$) inhibited the adherence of trypomastigotes to L 929 cells but had no effect on the adherence of the organisms onto normal or *C. parvum*-stimulated murine peritoneal MØ (Table 2).

Sialidase activity of T. cruzi trypomastigotes. Lysates of trypomastigotes

Table 2. Effect of treatment of L cells and murine macrophages on attachment of *T. cruzi* onto the cells

Cells	Treatment[a]	% of infection[b]	Parasites/infected cell
L 929	control	55±3	2.6±0.1
	VCNase	45±2	1.5±0.1**
Normal MØ	control	24±3	1.2±0.1
	VCNase	22±2	1.1±0.1
Elicited MØ	control	35±2	1.1±0.1
	VCNase	33±1	1.0±0.1

[a] Cell monolayers were established in plastic slides. After 24 h, the cells were fixed with p-formaldehyde and treated with 15 mU/well of VCNase or buffer alone (control) for 30 min at 37°C.

[b] A cell was defined as infected when parasites were demonstrated attached to the cell membrane. The results, representative of two experiments, are presented as mean ±S.D. from replicate cultures. Statistically significant differences between treated and control cells are presented as **($p<0.02$) (Student's t test).

Table 3. Sialidase activity in serum of mice acutely infected with different stocks of *T. cruzi*[a]

	Agglutination titer[b]	
T. cruzi	RBC[c]	sera[d]
Y	31	125
Tulahuén	62	500
MR	31	250
CL	31	125
Control	>1000	>1000

[a] Mice were bled 5 (Y, Tulahuén) or 8 (CL, MR) days after infection with 7×10^4 bloodstream trypomastigotes.

[b] The results are expressed as the minimal concentration of PNA (μg/ml) required to agglutinate the RBC.

[c] RBC were separated from blood, washed and assayed for agglutination in the presence of PNA.

[d] Serum was separated from blood and incubated with RBC from normal mice. After washing, the RBC were assayed for agglutination in the presence of PNA.

from either the Y, Tulahuén, MR, or CL stocks of *T. cruzi* desialized human RBC and cause the agglutination of these cells by PNA (data not shown).

Desialization of RBC and sialidase activity in acutely infected mice. RBC from mice infected with any of the four different stocks of *T. cruzi* were found to agglutinate in the presence of PNA (Table 3). The agglutination titers varied for each stock of *T. cruzi* but there was no correlation between the agglutination titers and either the parasitemias of the mice or the duration of the acute infection. No agglutination, however, was noted when the parasitemias were lower than 5×10^5 organisms per ml of blood.

Table 4. Effect of sera from mice acutely infected with *T. cruzi* on the infection of macrophages by bloodstream parasites

Experimental condition[a]	of infected cells[b]
Control medium	14.3±2.4[c]
10% NMS	15.1±0.5
10% Y serum	21.9±0.9* (+45)
10% CL serum	28.2±0.4** (+87)
50% NMS	14.5±1.4
50% Y serum	26.8±0.4* (+85)
50% CL serum	34.1±1.2** (+135)
50% CL (30 min×56°C	15.2±2.5 (+5)

[a] The entire assay was carried out by using medium supplemented with serum, in the proportions indicated, from normal mice (NMS), or from mice infected for 4 (stock Y) or 7 (stock CL) days with *T. cruzi*.

[b] Blood trypomastigotes of *T. cruzi* (stock Y) were used to infect monolayers of murine macrophages at a parasite:cell ratio of 3:1.

[c] The results, representative of six experiments, are presented as mean ±S.D.. Statistically significant differences with respect to the corresponding control (NMS) are presented as **($p<0.02$) or *($p<0.04$) (Student's t test). The percent increase is given in parenthesis.

Agglutination of normal mouse RBC by PNA was observed after incubation of the cells with sera from mice acutely infected with organisms of each one of the four stocks of *T. cruzi* (Table 3). Agglutination was observed with sera collected on day 4 to 7 post-infection (pi) from mice infected with the stocks Y or Tulahuén, and on day 6 to 10 pi in mice infected with the Cl or MR stocks. No significant differences in desialization activity was noted among the different stocks of *T. cruzi*. The ability of the sera to induce agglutination was abolished by heating at 56°C for 30 min.

Effect of sera from acutely infected mice on the interiorization of T. cruzi. Sera from mice acutely infected (4 to 7 days) with *T. cruzi* and devoid of specific antibodies to the organism detectable by ELISA were used to examine the interiorization process of bloodstream forms of *T. cruzi* into peritoneal MØ from normal mice (Table 4). Sera from mice infected either with the Y or CL stocks of *T. cruzi* significantly ($p<0.01$) increased the proportion of cells infected by Y parasites. The enhancement of interiorization of parasites by sera from acutely infected mice was abolished by heating the sera at 56°C for 30 min before the assay (Table 4). Similar enhancement of interiorization was caused by sera from mice infected with organisms of the stocks MR and Tulahuén (data not shown). Sera collected under identical conditions from mice similarly infected, however, did not reveal enhancing activity to the same extent. There was a wide variation; some sera failed to show any activity even when repeatedly tested.

Anti-sialidase activity in sera from mice and humans infected with T. cruzi. Humans and mice infected with *T. cruzi* developed anti-sialidase activity

Table 5. Effect of sera from mice or humans infected with *T. cruzi* on the capacity of VCNase to induce agglutination of human RBC[a]

Experimental condition	Agglutination titer[b]
Controls	16
NMS	16
Y	62
Tulahuén	125
MR	125
CL	62
NHS	16
Chronic (5 out of 7 sera)	>500
Chronic (2 out of 7 sera)	62
Acute-seronegative	31
Acute-seropositive	>500

[a] Normal human RBC were incubated with VCNase (500 U/ml) and PNA (sequential dilutions) in buffer only (controls), or in the presence (12.5% vol/vol) of serum from normal mouse (NMS); from mice infected for 6 (Y, Tulahuén) or 10 (Cl, MR) days with 7×10^4 bloodstream parasites; in the presence of normal human serum (NHS); sera from patients with chronic Chagas' disease (chronic) and two different samples of sera from an acutely infected patient, before (seronegative) and after (seropositive) seroconvertion.

[b] The results are expressed as the minimal concentration of PNA (μg/ml) required to agglutinate the RBC.

which inhibited the induction of agglutination of RBC by VCNase. In mice, this effect was noted in sera from animals infected with each one of the stocks of *T. cruzi* examined (Table 5). The anti-sialidase activity was demonstrated in serum of mice 7 days after their infection with Tulahuén or Y *T. cruzi* or 10 days after infection with CL or MR organisms. The ability of VCNase to induce agglutination or normal human RBC was abolished by 5 and reduced by 2 out of 7 sera from individuals with chronic Chagas' disease. Sera from normal individuals had no effect. In addition, serum samples from a patient with acute Chagas' disease failed to abolish the desialization of human RBC by VCNase while anti-*T. cruzi* serology was negative but completely abolished it when seroconversion occurred.

Discussion

The present study demonstrated sialidase activity in sera from mice acutely infected with each one of four different stocks of *T. cruzi*. Erythrocytes from these mice were agglutinated by PNA suggesting in vivo desialization.

Sialidase (neuraminidase) is a glycosidase able to cleave terminal sialic acid from heterosaccharides, glycolipids and glycoproteins (Gottschalk, 1957), regardless of whether they are free or part of the cell membrane surface (Seaman and Uhlenbruck, 1963). Neuraminidases are present in several pathogenic

microorganisms (Burnet and Stone, 1947; Hayano and Tanaka, 1969) but no clear role for these enzymes has been established yet. In some instances their presence has been associated with the severity of the desease caused (Kabir et al., 1984; Ogra et al., 1977).

The high sensitivity of different types of mammalian cells to sialidase activity (Haegert, 1979; Lauria and Catovsky, 1980; Smith et al., 1978; Suzuki et al., 1982) and the demonstration of neuraminidase activity in *T. cruzi* (Pereira, 1983a, b; Libby et al., 1986) suggested a role for this enzyme in the adsorption and subsequent penetration of the organism into host cells. Previous work to examine the effects of neuraminidase on the infection of mammalian cells by *T. cruzi* presented contradictory results (de Araujo Jorge and de Souza, 1984; Zenian and Kierszenbaum, 1982). This and the fact that the results reported in previous publications were obtained with neuraminidase from *Clostridium perfringens* which may contain protease and N-acetyl neuraminic-aldolase, both of them able to affect the final results, prompted us to further examine the effects of protease-free neuraminidase on the interaction of *T. cruzi* with mammalian cells. Our results revealed that treatment of blood stream trypomastigotes with up to 200 mU/ml of VCNase did not increase or decrease the ability of the organisms to invade phagocytic or non-phagocytic cells. These findings are in accord with previous observations (Crane and Dvorak, 1982) showing that sialic acid residues on the surface of *T. cruzi* trypomastigotes may not be involved in the adherence of these organisms to and penetration into certain mammalian cells.

Treatment of MØ from normal mice with 200 mU/ml of VCNase decreased the number of infected cells but the presence of 20 mU/ml of VCNAse during the entire period of infection of the cells resulted in enhancement of internalization of the organisms. Similar treatment of the non phagocytic L 929 cells, however, resulted in a consistent reduction in the number of internalized parasites. In addition, treatment of fixed L 929 cells with VCNase significantly inhibited the adherence of trypomastigotes to these cells but had no effect on the adherence of the organisms onto MØ. These results indicate that VCNase has different effects on the cell membrane surface of MØ and L 929 fibroblasts and suggest that sialic acid-containing glycoproteins in the cell membrane surface of these latter cells participate in the mechanism of recognition and interiorization of trypomastigotes by them. Interestingly, neuraminidase has been shown to induce similar reduction in the rate of attachment of *Leishmania tropica* amastigotes to cytochalasin-treated monocytes (Wyler and Suzuki, 1983) and of *Eimeria meleagrimitis* sporozoites to cultured turkey kidney cells (Augustine and Danforth, 1984). Taken together these data suggest that similar structures on the cell membrane surface of different mammalian cells are targets for protozoan attachment and invasion. The different effects of neuraminidase on MØ and on L 929 cells would indicate either that these particular sialic acid-containing residues are present with different densities on

the cell membrane surfaces of different cells and/or that the parasites recognize different structures on professional phagocytic and non phagocytic cells.

Our results showing that erythrocytes from mice infected with *T. cruzi* were agglutinated by PNA confirm previous observations (Pereira, 1983a) that desialization of red cells by a *T. cruzi*-neuraminidase occur in vivo. Because desialicated erythrocytes are liable to be removed from the circulation or lysed via the alternative pathway of activation of complement it has been suggested that the neuraminidase activity of *T. cruzi* may explain the anemia which frequently occurs in acute Chagas' disease (Libby et al., 1986).

Erythrocytes from normal mice were agglutinated by PNA following their treatment with sera from acutely infected mice suggesting the presence of sialidase in the sera. In addition, sera from acutely infected mice enhanced the interiorization of trypomastigotes within macrophages.

Of interest, however, was the observation that an anti-sialidase activity developed during the early stage of the infection. Thus, the ability of VCNase to induce agglutination of normal human RBC was inhibited by murine and human sera collected at some point early during the infection with *T. cruzi* when specific antibodies of the organism began to be detected by ELISA. In addition, induction of agglutination of RBC was also abolished or reduced by sera from 7 individuals chronically infected with *T. cruzi*. The results of these experiments do not provide any indication on the mechanism on the inhibitory effect and the possibility of anti-PNA activity in the inhibitory sera was not ruled out. However, the demonstration that *T. cruzi* releases neuraminidase in cultures (Pereira, 1983a) and our demonstration of neuraminidase activity in serum of acutely infected mice indicates that the development of anti-neuraminidase activity in individuals infected with *T. cruzi* seems likely. Our demonstration of anti-sialidase activity in serum of individuals chronically infected with *T. cruzi* further supports this possibility.

The results reported in this paper suggest that the sialidase activity of *T. cruzi* may play a role in the invasion of cells by the parasite during the very early stages of the infection. A mechanism to neutralize the sialidase activity, however, appears to develop rapidly and is effective during the subacute and chronic phases of the infection.

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