Glycobiology vol 2 no 6 pp 541–548, 1992

Substrate specificity of the *Trypanosoma cruzi* trans-sialidase

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***Trypanosoma cruzi* trypomastigotes acquire sialic acid (SA) from host glycoconjugates by means of a plasma membrane-associated trans-sialidase (TS). Here we study the substrate specificity of TS, which differs from all known sialyl-transferases in that it does not require cytidine monophosphate (CMP)-SA as donor. The *T.cruzi* TS reversibly transfers SA to saccharides with terminal β-Gal (but not α-Gal) residues. Donors are saccharides with SA linked to terminal β-Gal residues by (α2-3), but not (α2-6) bonds. The type of β-linkage of the terminal Gal residue is of minor importance (β1-4 and β1-6 are slightly better than β1-3), whereas chain length and the structure of additional vicinal sugar residues are not relevant. SA on the surface of living trypomastigotes of *T.cruzi* is transferred back and forth between the parasite surface and acceptor molecules with terminal β-Gal, either in solution or on the surface of neighbouring mammalian cells. Addition of fucose residue on or close to the terminal galactose impairs TS activity. As a consequence, the enzyme acts poorly on the E-selectin ligand sialyl-Lewis^x and its precursor Lewis^x, and *in vitro* adhesion of TS-treated neutrophils to L-cells expressing L-selectin is not affected. Modifications in the structure of the (α2-3)-linked *N*-acetyl-neuraminic acid (Neu5Ac) (deoxy or methoxy) of the donor molecules do not impair transfer if the changes are at C_9, whereas changes at C_4, C_7 and C_8 impair the ability to donate the modified SA. Compounds with modified C_4 and C_8 inhibit TS at relatively high inhibitor/substrate ratios.**

Key words: selectins/sialic acid/sialidase/trans-sialidase/ *Trypanosoma cruzi*

Introduction

Trypanosoma cruzi is the flagellated protozoan parasite that causes Chagas' disease, an incurable multisystemic disorder affecting millions of individuals in Latin America (Garcia-Zapata *et al.*, 1991). In the mammalian host, the amastigote forms of the parasite multiply in the cytoplasm of cells. After several cycles of replication, the amastigotes transform into flagellated trypomastigotes, which rupture the host cells and enter the circulation. The cycle is sustained when the trypomastigotes invade new cells. The attachment (Schenkman *et al.*, 1991b) and invasion (Schenkman *et al.*, 1992a) of mammalian cells by the trypomastigotes involves a surface membrane sialic acid (SA)-containing, stage-specific epitope (Ssp-3) (Andrews *et al.*, 1987). The sialylated form of Ssp-3 reacts with a panel of monoclonal antibodies (mAb) (Andrews *et al.*, 1987; Schenkman *et al.*, 1991b, 1992a), but removal of SA hinders recognition by the mAbs (Schenkman *et al.*, 1991a).

Trypanosoma cruzi does not synthesize SA (Schauer *et al.*, 1983), but acquires it from host glycoconjugates (Previato *et al.*, 1985; Zingales *et al.*, 1987) by means of a membrane trans-sialidase (TS) (Schenkman *et al.*, 1991a; Parodi *et al.*, 1992). This enzyme differs from all other known sialyl- and glycosyl-transferases in that it does not require a nucleotide-sugar as donor. Here we study the substrate specificity of the *T.cruzi* TS using biochemical and immunological approaches. In addition, we investigate the possibility that the enzyme may influence the migrating patterns of leukocytes by modifying the E-selectin ligands, sialyl-Lewis^x and sialyl-Lewis^a (Tyrrell *et al.*, 1991; Foxall *et al.*, 1992).

Results

SA acceptors

We initially examined the structural requirements for saccharides and glycolipids to accept SA in the reaction catalysed by TS. We assayed for the ability of these compounds to decrease the amounts of radiolabelled sialyllactose formed in a reaction mixture containing TS, 3′-sialyllactose as a donor of SA and [^{14}C]lactose as the acceptor. As shown in Table IA, transfer of SA to radiolabelled lactose decreases in the presence of disaccharides containing β-linked, but not α-linked terminal galactopyranosyl residues. Glc, GlcN, GalN, Fuc, GlcNac, GalNac, Man, and disaccharides containing GalNAc, Glc, GlcNAc and Man at the non-reducing end, are inactive. Gal is a poor acceptor (Table IC), but TS efficiently transfers SA to methyl-β-Gal, but not to methyl-α-Gal, confirming its specificity towards β-linked galactopyranosyl residues. Nevertheless, oligosaccharides containing non-terminal β-Gal residues are not good acceptors (see Table IB, 2′-FL and LNFP-I).

The reducing end of disaccharides influences the reaction since Gal(β1-4)GlcNAc and Gal(β1-6)GlcNAc are slightly better acceptors than Gal(β1-3)GlcNAc (Table IA and B). Chain length does not alter reactivity [compare LNnt with Gal(β1-4)GlcNAc, and LNT versus Gal(β1-3)GlcNAc, in Tables IA and B]. Addition of a fucose residue, however, significantly diminishes the capacity to inhibit formation of [^{14}C]3′-sialyllactose. The effect of the fucose residue decreases as it is positioned further away from the terminal Gal. While reactivity is almost totally blocked if the fucose residue is linked directly to the terminal Gal (see 2′-FL, LNFP-I, Table IB), the presence of branched fucose residues further away (compare LNFP-II and LNFP-V) is less inhibitory. Lewis^x (LNFP-III) is a poor acceptor (see Table IB).

We performed additional experiments using as a donor 4′-methyl-umbelliferyl *N*-acetyl-neuraminic acid (MU-Neu5Ac).

Table I. Ability of various compounds to decrease the TS-mediated formation of [sialyl-^{14}C]lactose. Results are expressed as percent reduction in the synthesis of [sialyl-^{14}C]lactose in a reaction mixture containing TS, 1 mM sialyllactose and 25 000–40 000 c.p.m. [D-glucose-1-^{14}C]lactose by adding different concentrations of potential acceptor molecules

(A) Disaccharides and derivatives

Saccharides	Name	Concentration (μM)[a]		
		100	1000	10 000
Gal(β1-4)Glc	Lactose	26	76	86
Gal(β1-4)fructose	Lactulose	–	59	78
Gal(β1-4)gluconic acid	Lactobionic acid	26	50	74
Gal(β1-4)GlcNAc	*N*-Acetyl-lactosamine	36	76	89
Gal(β1-4)Man		–	36	71
Gal(β1-4)Glc-O-CH_3		42	61	77
Gal(β1-3)GlcNAc		–	–	52
Gal(β1-3)GalNAc		5	28	55
Gal(β1-3)arabinose		7	37	66
Gal(β1-3)Gal-O-CH_3		18	67	78
Gal(β1-6)Gal		–	38	83
Gal(β1-6)GlcNAc		–	40	89
Gal(α1-4)Gal		–	–	–
Gal(α1-3)Gal-O-CH_3		6	4	13
Gal(α1-6)Glc	Melibiose	–	–	–
Gal(α1-6)Gal	Stachyose	–	–	–
GalNAc(β1-3)Gal-O-CH_3		–	1	–
Glc(β1-4)Glc	Cellobiose	–	–	–
Glc(α1-6)fructose	Palatinose	–	–	–
Glc(α1-4)Glc	Maltose	–	–	–
Glc(α1-1β)fructose	Sucrose	–	–	–
Glc(α1-1α)Glc	α,α-Trehalose	–	–	–
GlcNAc(β1-3)Gal-O-CH_3		–	–	–
GlcNAc(β1-6)Gal		–	–	–
GlcNAc(β1-4)GlcNAc	Chitobiose	–	–	–
GlcNAc(β1-6)GlcNAc		–	–	1
GlcNAc(β1-6)Man-O-CH_3		–	–	4
Man(α1-3)Man		–	–	–

[a]–, no reduction.

(B) Oligosaccharides

Name	Saccharides	Concentration (μM)		
		100	1000	10 000
LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	12	69	84
LNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	1	13	69
2′-FL	Fuc(α1-2)Gal(β1-4)Glc	6	–	12
3-FL	Gal(β1-4)[Fuc(α1-3)]Glc	–	11	61
LNFP-I	Fuc(α1-4)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	–	8	28
LNFP-II	Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)Glc	–	12	21
LNFP-III[a]	Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc	–	18	ND
LNFP-V	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]Glc	9	–	52

[a]'Lewis'.

(C) Monosaccharides and derivatives[a]

Name	Concentration (μM)		
	100	1000	10 000
Galactose	–	7	20
α-Methyl-galactose	13	19	17
β-Methyl-galactose	26	37	81

[a]Glucose (Glc), glucosamine (GlcN), galactosamine (GalN), fucose (Fuc), *N*-acetyl-glucosamine (GlcNAc), *N*-acetyl-galactosamine (GalNAc) and mannose (Man) were all tested and found to be negative.

All β-linked galactosides are sialylated by TS in the presence of MU-Neu5Ac (Figure 1, lanes C, D and E), whereas Gal(β1-4)[Fuc(α1-3)]Glc (3′-FL, lane B) is only poorly sialylated. Note that in the absence of SA acceptors (lane F), or in the presence of saccharides which are not good acceptors (lane B), free SA is generated. This confirms that under these conditions the enzyme acts more as a neuraminidase [better called sialidase (Reuter and Schauer, 1988)] than as a trans-sialidase (Schenkman *et al.*, 1992b).

SA donors

Next, we assayed for the ability of various sialylated molecules to act as donors of SA to [^{14}C]lactose. As shown in Table II, (α2-3)-linked, but not (α2-6)- or (α2-9)-linked SA is transferred to [^{14}C]lactose. In parallel with the results above (Table I), the saccharides in which Gal is (β1-4) linked are better SA donors than those in which Gal is (β1-3) linked (compare GSC-31 and GSC-30, Table IIA). The presence of fucose linked to the terminal Gal hampers donation of SA (compare 3′-sialyllactose with 3′-S,3-FL, Table IIB), but if the fucose is attached to the penultimate residue, there is some transfer (as in sialyl-Lewis^x, Table IIA). Table IIC shows that transfer only occurs if SA is (α2-3) linked to terminal and not internal galactopyranosyl residues. Table IIB further demonstrates that any sialylated terminal β-Gal, whether O- or N-linked type oligosaccharides, are potential donors.

TS inhibitors

In Table IID we studied the effect of structural changes in the neuraminic acid residue of GM_3-gangliosides which, as expected, are good SA donors. Modifications at C_9 (deoxy or methoxy) do not alter the ability of the gangliosides to donate SA, whereas changes at C_4 (deoxy or methoxy), C_7 (deoxy) and C_8 (deoxy or epi) are profoundly inhibitory. In addition, Table III shows that some of these compounds are in fact inhibitors of TS activity. GSC-77, GSC-75 and GSC-50 (but not the other gangliosides) partially prevent the transfer of SA from 3′-sialyllactose to [^{14}C]lactose at 1/10 inhibitor/substrate ratios.

The inhibitory activity of the compounds was also shown *in vivo*. At relatively high concentrations GSC-84, the saccharide portion of GSC-77, partially inhibits sialylation of trypomastigotes grown in bovine serum albumin (BSA) when incubated with 3′-sialyllactose (Table IV). GSC-84 was used instead of GSC-77 because the ceramide portion of GSC-77 caused aggregation of the trypomastigotes (data not shown). Nevertheless, complete inhibition of TS was not achieved with any of the gangliosides, thus rendering them of little use for biological experiments. Table III also shows that 2,3-dehydro-2-deoxy-Neu5Ac, a potent inhibitor of bacterial and viral sialidases (Meindl *et al.*, 1974), has little or no effect on TS activity.

Ssp-3 is a donor of SA

Previous studies demonstrated that the Ssp-3 epitope of trypomastigotes (Andrews *et al.*, 1987) is assembled through the TS-catalysed transfer of (α2-3)-linked SA from host macromolecules to parasite surface molecules (Schenkman *et al.*, 1991a). To determine whether this reaction is reversible, and whether Ssp-3 can in turn donate SA to other cells, we mixed parasites grown in Dulbecco's modified Eagle's medium (DMEM)–BSA with parasites grown in DMEM–fetal bovine serum (FBS) and incubated the mixture for 60 min at 37°C. The mixture was then subjected to FACS analysis using mAb 3C9 as a revealing reagent. The reactivity of this mAb with Ssp-3 is dependent on the presence of SA in the epitope. As shown in Figure 2, parasites grown in DMEM–BSA (therefore in the absence of SA donors) (A) are less intensely stained (mean fluorescence = 242) than those grown in medium containing FBS (B) (mean fluorescence = 1371). After they are incubated together, the parasites have intermediate staining intensity (C) (mean fluorescence = 1090).

That Ssp-3 can act as a donor of SA was further demonstrated by incubating parasites grown in DMEM–FBS in medium containing 1 mM of various oligosaccharides. The Ssp-3 epitope is lost if the medium contains saccharides with terminal β-Gal residues, such as lactose and β-methyl-Gal, but not if it contains saccharides with α-terminal Gal residues, such as melibiose or α-methyl-Gal (see Table V).

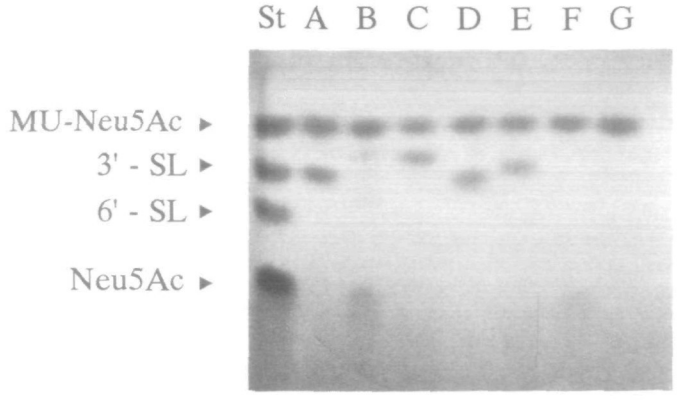

Fig. 1. Saccharides with terminal β-linked Gal are acceptors for TS. Affinity purified TS was incubated for 3 h at room temperature with MU-Neu5Ac and different oligosaccharides (**lane A**: lactose; **B**: 3-FL; **C**: Gal(β1-3)GlcNAc; **D**: Gal(β1-6)GlcNAc; **E**: Gal(β1-3)GalNAc) as indicated. In the control reaction mixtures, TS (**lane F**) or heat-inactivated TS (**lane G**) was incubated with MU-Neu5Ac alone. The reactions were stopped by adding ethanol and the products were analysed by TLC on silica gel 60 plates in ethanol–*n*-butanol–pyridine–water–acetic acid [100:10:10:30:3 (v/v)]. SA-containing molecules were visualized by resorcinol staining. MU-Neu5Ac, 3′-sialyllactose, 6′-sialyllactose and Neu5Ac were used as standards (arrows).

Lack of TS effect on E-selectin ligand

Sialic acid plays an important role in the selectin-mediated cell adhesion (Tyrrell *et al.*, 1991; Foxall *et al.*, 1992). Additional experiments were performed to examine the effect of TS on the interaction between selectins and their ligands, sialyl-Lewis^a and sialyl-Lewis^x. Neutrophils were treated with TS in the presence of lactose (as a SA acceptor), or with *Vibrio cholerae* sialidase (as a positive control). The neutrophils were then assayed for their ability to attach to L-cells transfected with E-selectin. Treatment with sialidase, but not with TS, abrogated the binding (Figure 3).

In order to document the activity of TS on SA-containing molecules from the neutrophil plasma membrane, we performed a series of control experiments. Neutrophils were pre-incubated with phosphate-buffered saline (PBS), with sialidase, or with TS in the presence of lactose. The cells were washed and reincubated with TS in the presence of [D-glucose-1-^{14}C]-lactose. Negative controls consisted of cells incubated with the labelled acceptor, but in the absence of TS. As shown in Table VI, labelled sialyllactose is formed in the incubation mixtures containing neutrophils which had been pre-treated with PBS,

Table II. Ability of various compounds to be sialic acid donors. [Sialyl-^{14}C]lactose formation in a reaction mixture containing TS, [D-glucose-1-^{14}C]lactose and different concentrations of potential donor molecules was expressed as a percentage of c.p.m.s obtained in the presence of 1 mM of a reference donor (different for every table)

(A) Gangliosides

Name	Terminal saccharides	Concentration (μM)[a]		
		10	100	1000
GSC-31	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-R[b]	71	91	100
GSC-30	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc-R	23	42	55
GSC-64	Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Glc(β1-4)Gal-R, sialyl-Lewis^x	12	19	42
GSC-61	Neu5Ac(α2-6)Gal(β1-4)Glc-R	–	18	10
GSC-65	Neu5Ac(α2-6)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc-R	3	9	1
GSC-96	Neu5Ac(α2-9)SA(α2-3)Gal(β1-4)Glc-R	8	13	11

[a]Results are expressed as a percentage of c.p m. formed in the presence of 1 mM GSC-31.
[b]R= -O-CH_2-CH(NH-CO-$CO_{17}H_{35}$)-CHOH-CH=CH$C_{13}H_{27}$

(B) Oligosaccharides

Name	Terminal saccharides	Concentration (μM)[a]			
		10	100	1000	10 000
3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc	36	63	100	
6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc	18	21	18	
LST-a	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	0	2	31	71
3′-S,3-FL	Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]Glc	3	2	8	
C-446300	N-linked type oligosaccharide	53	89		

[a]Results are expressed as a percentage of c.p.m. formed in the presence of 1 mM 3′-SL.

(C) Gangliosides with terminal and non-terminal SA

Name	Terminal saccharides	Concentration (μM)[a]		
		10	100	1000
Monosialo-GM_1	Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-4)Glc-Cer	5	6	6
Monosialo-GM_2	GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-34)Glc-Cer	6	10	10
Monosialo-GM_3	Neu5Ac(α2-3)Gal(β1-4)Glc-Cer	52	122	100

[a]Results are expressed as a percentage of c.p.m. formed in the presence of 1 mM GM_3

(D) GM_3-gangliosides [Neu5Ac(α2-3)Gal(β1-4)Glc-R] with variations in the Neu5Ac residue

Name	Variations in Neu5Ac	Concentration (μM)[a]		
		10	100	1000
GSC-17	Neu5Ac	18	86	100
GSC-50	8-epi Neu5Ac	–	1	5
GSC-51	9-deoxy Neu5Ac	32	88	107
GSC-75	4-deoxy Neu5Ac	1	5	9
GSC-76	7-deoxy Neu5Ac	0	1	4
GSC-77	8-deoxy Neu5Ac	5	9	9
GSC-78	4-methoxy Neu5Ac	1	3	58
GSC-79	9-methoxy Neu5Ac	24	71	71

[a]Results are expressed as a percentage of c.p.m. formed in the presence of 1 mM GSC-17 (GM_3).

but not with sialidase, or with TS in the presence of lactose. We conclude that TS removes (α2-3)-linked SA from the plasma membrane of the neutrophils. However, the E-selectin ligands, sialyl-Lewis^x and sialyl-Lewis^a, that contain branched fucose, are less (or not) affected.

Discussion

Glycosyl transfer reactions are common in nature, the most common type being the transfer of a glycosyl unit from a sugar nucleotide to an acceptor. Transglycosylation in the absence of nucleotide-sugars is unusual because this process is thermodynamically unfavourable (Cote and Tao, 1990). Nevertheless, many enzymes that primarily catalyse the hydrolysis of oligosaccharides can perform transglycosylation reactions, albeit much less efficiently. The hydrolysis of the glycosidic bond by these enzymes is simply a special case of transglycosylation, in which the acceptor is water. This is also the case for the *T.cruzi* TS, whose sialidase activity (Pereira, 1983) was detected prior to its trans-sialidase activity (Schenkman *et al.*,

Table III. Inhibition of transfer of SA by 2,3-dehydro-2-deoxy-Neu5Ac and gangliosides. 100 μM 3′-sialyllactose, [^{14}C]lactose and TS were incubated with sialyl-saccharides at different concentrations for 3 min at room temperature. Inhibition is expressed as percentage reduction of c.p.m. obtained in the absence of the sialyl-saccharides

SA-saccharide[a]	Concentration (μM)[b]			
	1	10	100	1000
2,3-dehydro-2-deoxy-Neu5Ac	3	–	–	9
GSC-77	–	44	79	90
GSC-75	–	46	87	80
GSC-50	12	24	60	84
GSC-76	–	–	–	52
GSC-96	–	–	–	9
GSC-61	–	–	–	25

[a]The substances tested do not transfer sialic acid to [^{14}C]lactose (see Table II)
[b]–, no inhibition.

Table IV. Inhibition of sialylation of trypomastigotes by GSC-84 Trypomastigotes were grown in DMEM-BSA and incubated with 3′-sialyllactose (0.01 mM) or 3′-sialyllactose + GSC-84 (0.1 mM) for 5 or 30 min at 37°C, stained with an antibody recognizing a sialylated epitope (mAb 3C9) and subjected to FACS analysis

Trypomastigotes incubated with	Mean fluorescence after	
	5 min	30 min
DMEM–BSA	234	180
DMEM–BSA + SL	983	1377
DMEM–BSA + SL + GSC-84	674	695

1991a). However, the *T.cruzi* enzyme is exceptional in that it is more efficient in transferring than in hydrolysing SA (Parodi *et al.*, 1992; Schenkman *et al.*, 1992b). Also, in contrast with the glycosyltransferases, it does not use CMP-SA (which contains β-linked SA) as a donor of SA (Corfield and Schauer, 1982).

In this paper, we characterize the substrate specificity of the *T.cruzi* TS using biochemical and immunological approaches. We show that saccharides with terminal β-Gal (but not α-Gal) are acceptors, and saccharides with terminal Neu5Ac (α2-3)-linked to β-Gal [but not (α2-6)-linked], are donors of SA (Tables I and II). The presence of a residue in β-linkage to Gal is necessary for catalysis; Gal is not an acceptor, although in aqueous solutions Gal occurs mostly as a β-anomer (El Khadem, 1988). On the other hand, a single linked carbon atom (methyl-β-Gal) is sufficient to obtain reactivity (Table I). TS also transfers SA from MU-Neu5Ac, but methyl-umbelliferone is not an acceptor (Schenkman *et al.*, 1992b). A possible interpretation of these findings is that during catalysis a sialylated enzyme intermediate is formed and the desialylated product of the reaction is released from the pocket. The enzyme-bound SA is then transferred to any available β-Gal-containing structure, provided it can be accommodated in the enzyme pocket. Although chain length and the structure of additional vicinal sugar residues seem irrelevant, addition of a fucosyl side chain to the terminal β-Gal inhibits transfer, most likely because it prevents entrance of the saccharide in the active site of TS (Tables I and II), as in the case of mammalian sialyl-transferases (Paulson *et al.*, 1978).

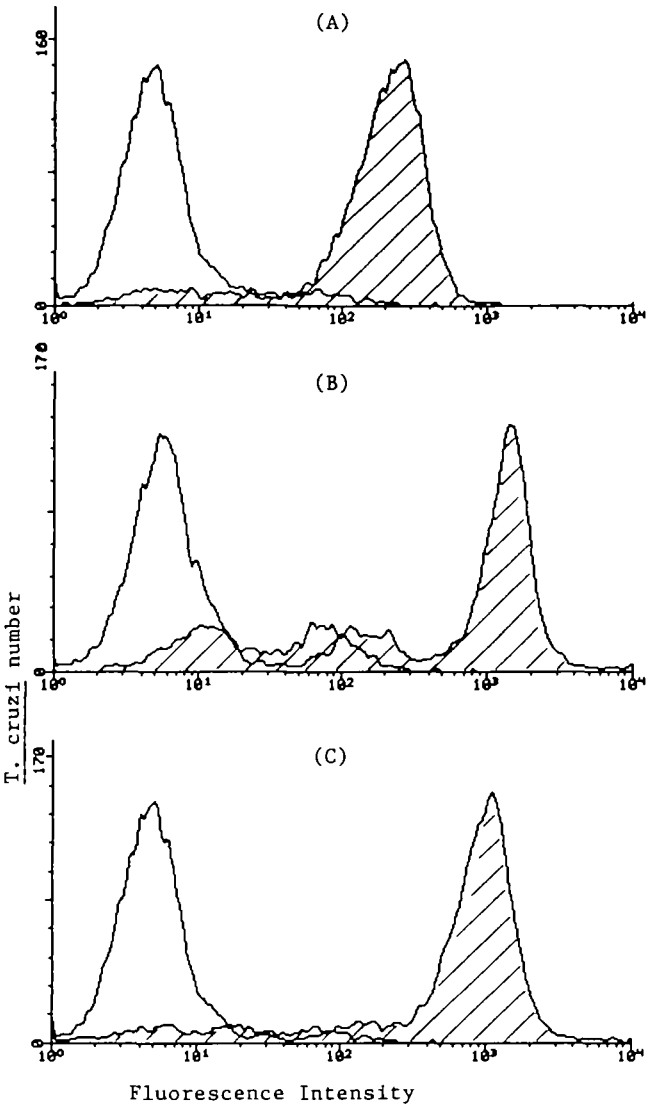

Fig. 2. The acquisition of SA by trypomastigotes is reversible. Parasites were washed in DMEM–BSA and divided into two aliquots. One was incubated with the mAb 3C9 against the Ssp-3 epitope. Parasites were then stained with goat anti-mouse IgG–FITC and analysed by FACS. Three different samples of trypomastigotes were analysed, originating from cultures in DMEM–BSA (**A**), cultures in DMEM–FBS (**B**), or a mixture of equal numbers of (A) and (B) which was pre-incubated at 37°C for 60 min prior to staining (**C**). Unshaded curves represent the background obtained by staining with the secondary antibody only; shaded curves represent the 3C9-stained samples.

Table V. Ssp-3 is a donor of sialic acid. Trypomastigotes were grown in medium containing BSA or FCS as described. Parasites were then incubated with different saccharides (1 mM) for 1 h at 37°C, stained with the 3C9 antibody and analysed by FACS

Trypomastigotes grown in	Additional incubation in	Mean fluorescence
BSA	PBS	234
FCS	PBS	1905
	Lactose	746
	Melibiose	1649
	βGal-O-CH_3	848
	αGal-O-CH_3	1845

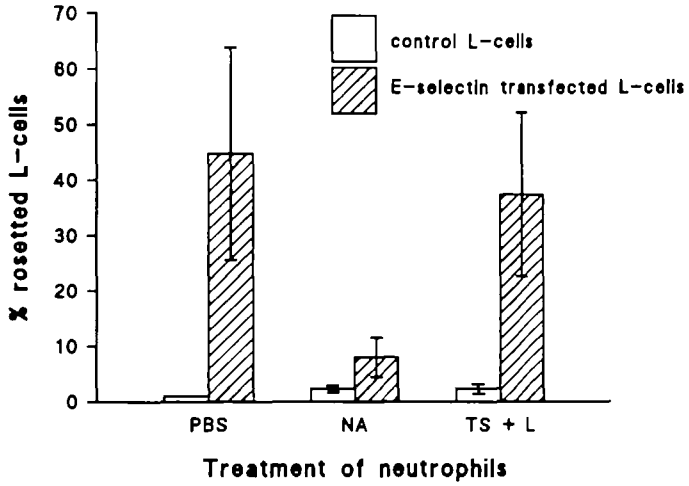

Fig. 3. TS does not affect the binding of neutrophils to L-cells transfected with E-selectin. Polymorphonuclear leukocytes were washed three times with DMEM and resuspended in DMEM–Pipes (50 mM, pH 6.5). Cells (8×10^6) were treated with 0.2 U/ml *V.cholerae* sialidase, with purified TS in the presence of 1 mM lactose, or with an equal volume of PBS. Neutrophils were incubated at 37°C for 90 min, spun down and washed twice with DMEM. Cultures of L-cells transfected with E-selectin cDNA were washed once with DMEM without FBS, and co-incubated with 2 ml of the neutrophil suspension at 37°C on a rotary shaker. The medium was removed after 60 min and the cells washed six times with DMEM without FBS. Rosetting of neutrophils around the adherent L-cells was evaluated microscopically and expressed as percentage of L-cells with more than one neutrophil attached.

Table VI. TS-mediated transfer of sialic acid from neutrophils Neutrophils (7×10^6 cells) were pre-treated with PBS, with sialidase or with TS + lactose, and then incubated in DMEM-Hepes (50 mM, pH 7.2) with TS and [D-glucose-1-^{14}C]lactose for 90 min at room temperature on a rotary shaker. The supernatants were then passed over a QAE column and radioactivity measured as described in Materials and methods

Neutrophils pre-treated with	c.p.m. retained on the QAE column
PBS	945
TS + L	58
Sialidase	80
PBS[a]	125

[a]Neutrophils were incubated with [D-glucose-1-^{14}C]lactose in the absence of TS.

As shown in Table III, the compounds with modified C_4 and C_8 (deoxy) in the (α2-3)-linked Neu5Ac are inhibitors of TS, suggesting that these are sites for SA recognition/catalysis. This is in contrast with the saccharides bearing (α2-6)- or (α2-9)-linked SA, which are neither donors nor inhibitors and probably do not enter the enzyme pocket.

Every donor molecule tested (with the exception of MU-Neu5Ac, see above) can become an acceptor after release of SA (Tables I and II). This is also valid for Ssp-3, the sialylated stage-specific epitope of *T.cruzi* (Andrews *et al.*, 1987). Studies performed with live trypomastigotes show that SA can be transferred back and forth between the parasite surface and acceptor molecules (Figure 2, Tables V and VI). It is conceivable that this continuous transfer of SA might in fact be part of the molecular events occurring during parasite attachment to and invasion of host cells (Schenkman *et al.*, 1991b).

On the basis of the initial studies on the substrate specificity of TS, it was suggested (Kahn *et al.*, 1991; Schenkman *et al.*, 1991a) that TS might remove the SA from the selectin ligands [sialyl-Lewis^x and sialyl-Lewis^a (Tyrrell *et al.*, 1991; Foxall *et al.*, 1992)]. If this were true, the TS shed from the parasite surface (Frevert *et al.*, 1992b; Schenkman *et al.*, 1992), and found in the circulation during the acute stages of the infection (de Titto and Araujo, 1988; Affranchino *et al.*, 1989), might disrupt specific interactions between leukocytes, platelets and the vascular endothelium. However, the data in Table II, showing that fucosylated saccharides are not very good substrates for the TS, render this unlikely. The biochemical data were substantiated by direct experiments showing that *in vitro* adhesion of TS-treated neutrophils to L-cells expressing L-selectin was not significantly affected (Figure 3).

TS is a homopolymer with subunits of 160 000–200 000 M_r, with the amino-terminal half of the polypeptide chain containing the catalytic site (Parodi *et al.*, 1992; Uemura *et al.*, 1992). This region of the molecule contains consensus sequences (S-X-D-X-G-X-T-W) which are homologous to the ASP boxes in the viral and bacterial sialidases (Roggentin *et al.*, 1989; Russo *et al.*, 1990; Kahn *et al.*, 1991; Pollevick *et al.*, 1991; Uemura *et al.*, 1992). In spite of these similarities, TS has properties distinct from the other sialidases. TS is most active at neutral pH, whereas most sialidases are more active at acidic pH. Competitive inhibitors of sialidases, such as 2,3-dehydro-3-deoxy-Neu5Ac, do not inhibit TS (Table III). In fact, ASP boxes are found in proteins that do not have sialidase activity, but are involved in binding to saccharides (Takle and Cross, 1991). Similarly, ASP boxes are found in trypomastigote surface molecules lacking sialidase activity (Takle and Cross, 1991; Uemura *et al.*, 1992). Perhaps ASP boxes function in the recognition of saccharides, rather than in catalysis, and may be required to attach *T.cruzi* trypomastigotes to cells or to the extracellular matrix. The recent cloning of several members of the TS family of genes, some of which lack TS activity (Uemura *et al.*, 1992), should facilitate the identification of domains of the polypeptide chain involved in the recognition of donors and acceptors of SA, as well as in catalysis.

Materials and methods

Parasites

Trypanosoma cruzi trypomastigotes, Y strain (Silva and Nussenzweig, 1953), were obtained from infected LLC-MK_2 cells (ATCC-CCL-7, Rockville, MD), grown in low-glucose DMEM with penicillin and streptomycin (Gibco, Grand Island, NY), containing 10% FBS. Subconfluent cultures of LLC- MK_2 cells, kept at 37°C in 5% CO_2, were infected with 5×10^6 trypomastigotes/75 cm^2 flask. The parasites remaining extracellular after 24 h were removed and the cultures maintained in 10% FBS–DMEM. In some experiments, the FBS-containing medium was removed during the third day following infection, the monolayers washed twice with PBS, and culture continued in DMEM containing 0.2% BSA (ultrapure, Boehringer Mannheim, Indianapolis, IN) and 20 mM Hepes (pH 7.4) (0.2% BSA–DMEM). There was no difference in the numbers or morphology of parasites obtained from cultures in 0.2% BSA–DMEM (BSA-trypomastigotes) or in FBS–DMEM (FBS-trypomastigotes). On day 5 after infection, the culture supernatants containing trypomastigotes, intermediate forms and amastigotes were centrifuged at 2000 *g* for 5 min and incubated at 37°C. The motile trypomastigotes were collected from the supernatant after 2 h.

Enzyme purification

TS activity was purified as described previously (Schenkman *et al.*, 1992b), with slight modifications. Pooled supernatants from cultures of BSA-trypomastigotes were passed through a 0.22 μm Millipore filter and then concentrated ~20 times by filtration through Amicon membranes with a 300 000 mol. wt

cutoff (XM300, Amicon Co, Lexington, MA). The supernatants were adjusted to 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and incubated with Concanavalin A–Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 0.1% NP-40, 0.5 M NaCl, 50 mM Tris–HCl (pH 7.4). The Sepharose beads were washed with 25 ml of the equilibration buffer and the enzyme eluted by overnight incubation of the beads with 0.5 M α-methyl-D-mannoside in the same buffer. The eluate was then dialysed against PBS.

Alternatively, the material concentrated by passage through Amicon membranes was incubated with mAb 39 (Schenkman *et al.*, 1991b) immobilized on a tresyl-activated agarose column (Schleicher & Schuell, Keene, NH), prepared according to the manufacturer's instructions. The column was washed with 0.15 M NaCl, 50 mM Tris–HCl (pH 7.4), 0.05% NP-40, and the enzyme eluted with 3.5 M $MgCl_2$, 20 mM sodium phosphate (pH 6.0). The eluate was immediately filtered through a G-25 column equilibrated with 20 mM Tris–HCl (pH 8.0).

Enzyme activity measurements

TS activity was assayed using two different procedures. (i) By incubating purified enzyme in 20 mM Hepes buffer (Sigma H-3375) (pH 7.2) in the presence of 3′-sialyllactose and [D-glucose-1-^{14}C]lactose (60 mCi/mmol) (Amersham, Arlington Heights, IL) as described previously (Passaniti and Hart, 1988). A standard assay contained 1 mM 3′-sialyllactose and 25 000–40 000 c.p.m lactose in a final volume of 50 μl. This mixture was incubated for 30 min at 37°C and the reaction terminated by the addition of 1 ml of water, followed by passage through a 1 ml QAE–Sephadex A50 column equilibrated in water (Pharmacia LKB). The radioactive oligosaccharides were eluted with 0.5 ml of 1 M ammonium formate. The effectiveness of enzyme acceptors or inhibitors was assayed by adding them at various concentrations to a standard reaction mixture. The ability of different sialylated molecules to donate their SA was evaluated by substituting them in the reaction mixture for 3′-sialyl lactose at various concentrations. Activity was expressed as percent reduction of control counts for the acceptor/inhibitor studies, or as percent of control counts for the donor as compared to a standard reaction run in parallel. (ii) Transfer activity was also measured by incubating affinity purified TS with 50 nmol of MU-Neu5Ac and 40 nmol of the indicated oligosaccharide, in 20 μl 5 mM Hepes (pH 7) for 3 h at room temperature. In the control reactions, TS or heat-inactivated TS were incubated with MU-Neu5Ac alone. The reactions were stopped by adding 20 μl ethanol and the products analysed by TLC on silica gel 60 plates (EM Science, HPTLC Fertigplatten Kieselgel 60F254, 10 × 10 cm) and chromatographed in ethanol–*n*-butanol–pyridine–water–acetic acid [100:10:10:30:3 (v/v)]. SA-containing molecules were visualized by resorcinol staining. Neu5Ac, MU-Neu5Ac, 3′-sialyllactose and 6′-sialyllactose were used as standards.

Saccharides, ceramides and glycoproteins

All products were from Sigma, except those marked OG which were purchased from Oxford Glycosystem; or those marked BM, which were purchased from Boehringer Mannheim; or those synthesized at Gifu University, indicated by a reference.

Sialic acid acceptors. (i) Monosaccharides: D-Glucose, DGlc; D-Glucosamine, 2-Amino-2-deoxy-D-Glucose, DGlcN; D-Gal, DGal; D-Galactosamine, 2-Amino-2-deoxy-D-Gal, DGalN; D-Mannose, D-Mannopyranose, DMan; L-Fucose, LFuc; *N*-Acetyl-D-Glucosamine, D-GlcNAc; *N*-Acetyl-D-Galactosamine, D-GalNAc; α-Methyl-D-Galactopyranoside; β-Methyl-D-Galactopyranoside. *(ii) Disaccharides*: Lactose, 4-*O*-β-D-Galactopyranosyl-α-D-Glucose, DGal(β1-4)DGlc; *N*-Acetyl-Lactosamine, 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucopyranose, DGal(β1-4)DGlcNAc; 2-Acetamido-2-deoxy-6-*O*-β-D-galactopyranosyl-D-glucopyranose, D-Gal(β1-6)DGlcNAc; 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-glucopyranose, D-Gal(β1-3)DGlcNAc; 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactopyranose, DGal-(β1-3)DGalNAc; 6-*O*-β-D-Galactopyranosyl-D-Gal, DGal(β1-6)DGal; 4-*O*-α-D-Galactopyranosyl-D-Galactopyranose, DGal(α1-4)DGal; Methyl 3-*O*-α-D-Galactopyranosyl-α-D-Galactopyranoside, DGal(α1-3)DGal-*O*-CH_3; Methyl 3-*O*-β-D-Galactopyranosyl-β-D-Galactopyranoside, DGal(β1-3)DGal-*O*-CH_3; Methyl 4-*O*-β-D-Galactopyranosyl-β-D-Glucopyranoside, DGal(β1-4)DGlc-*O*-CH_3; 4-*O*-β-Galactopyranosyl-D-Mannopyranose, DGal(β1-4)DMan; 3-*O*-α-D-Mannopyranosyl-D-Mannopyranose, DMan(α1-3)DMan; 3-*O*-β-D-Galactopyranosyl-D-Arabinose, DGal(β1-3)DAra; 2-Acetamido-6-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose, DGlcNAc(β1-6)-GlcNAc; 6-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-D-galactopyranose, DGlcNAc(β1-6)DGal; Methyl-3-*O*-(2-Acetamido-2-deoxy-β-D-galactopyranosyl)-α-D-galactopyranoside, DGalNAc(β1-3)DGal-*O*-CH_3; Methyl-3-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside, DGlcNAc-(β1-3)DGal-*O*-CH_3; Methyl-6-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranoside, DGlcNAc(β1-6)DMan-*O*-CH_3; Lactulose, 4-*O*-β-D-Galactopyranosyl-D-fructofuranose, DGal(β1-4)DFru; Lactobionic Acid, 4-*O*-β-D-Galactopyranosyl-D-gluconic acid, DGal(β1-4)DGlcNAc; Palatinose, 6-*O*-α-D-Glucopyranosyl-D-fructofuranose, DGlu(α1-6)DFru; D-(+)-cellobiose, 4-*O*-β-D-glucopyranosyl-D-glucopyranosyl, DGlc(β1-4)DGlc; D-(+)-trehalose, α,α-Trehalose, α-D-Glucopyranosyl-α-D-glucopyranoside; Maltose, 4-*O*-α-D-Glucopyranosyl-D-Glucose, DGlc(α1-4)DGlc; Melibiose, 6-*O*-α-D-Galactopyranosyl-D-Glucose, DGal(α1-6)DGlc; Sucrose, α-D-Glucopyranosyl-β-D-fructofuranoside, Glc(α1-1β)fructose. *(iii) Oligosaccharides*: 2′-Fucolactose, 2′-FL, LFuc(α1-2)DGal(β1-4)DGlc, OG; 3-Fucolactose, 3-FL, DGal(β1-4)-DGlc(α3-1)LFuc, OG; Lacto-*N*-neotetraose, LNnT, DGal(β1-4)DGlcNAc-(β1-3)DGal(β1-4)DGlc, OG; Lacto-*N*-tetraose, LNT, DGal(β1-3)DGlcNAc-β1-3)DGal(β1-4)DGlc, OG; Lacto-*N*-fucopentaose I, LNFP-I, LFuc(α1-4)-DGal(β1-3)DGlcNAc(β1-3)DGal(β1-4)DGlc, OG; Lacto-*N*-fucopentaose II, LNFP-II, DGal(β1-3)DGlcNAc[LFuc(α1-4)](β1-3)DGal(β1-4)DGlc, OG, Lacto-*N*-fucopentaose III, LNFP-III, DGal(β1-4)DGlcNAc[LFuc(α1-3)]-(β1-3)DGal(β1-4)DGlc, OG; Stachyose, α-D-Gal-[1-6]-α-D-Gal-[1-6]-α-D-Glc-[1-2]-β-D-Fru.

Sialylated compounds. (i) Monosaccharides: 2,3-Dehydro-2-Deoxy-*N*-acetyl-neuraminic Acid, BM. *(ii) Oligosaccharides*: 3′-Sialyllactose, SL, *N*-Acetyl-neuraminosyl-D-Lactose, Neu5Ac(α2-3)DGal(β1-4)DGlc, OG; 6′-Sialyllactose, SL, *N*-Acetyl-neuraminosyl-D-Lactose, Neu5Ac(α2-6)DGal(β1-4)DGlc, OG; LS-Tetrasaccharide$_a$, LST-a, Neu5Ac(α2-3)DGal(β1-3)DGlcNAc(β1-3)DGal-(β1-4)DGlc, OG; 3′-Sialyl-3-fucosyllactose, 3′-S,3-FL, Neu5Ac(α2-3)DGal-(β1-4)DGlc[DFuc(α1-3)], OG; tetra-sialylated, galactosylated tetra-antennary complex N-linked-type oligosaccharide, containing four terminal Neu5Ac-(α2-3)Gal(β1-4)GlcNAc residues per molecule, OG C-446300. *(iii) Gangliosides*: (R_1 = -CH_2-CH(NH-CO-$C_{17}H_{35}$)-CHOH-CH=CH-$C_{13}H_{27}$, Cer = Ceramide); Sialyl-(α2-6)-Lacto-*N*-tetraosylCer, Neu5Ac(α2-6)DGal(β1-3)-DGlcNAc(β1-3)DGal(β1-4)DGlc-O-R1, GSC-65 (Hasegawa *et al.*, 1991); Sialyl-Lewis^x-Cer, Neu5Ac(α2-3)DGal(β1-4)[DFuc(α1-3)]DGlcNAc(β1-3)-DGal(β1-4)DGlc-O-R_1, GSC-64 (Kameyama *et al.*, 1991); Sialyl-Lacto-*N*-tetraosylCer, Neu5Ac(α2-3)DGal(β1-3)DGlcNAc(β1-3)DGal(β1-4)DGlc-O-R_1, GSC-30 (Kameyama *et al.*, 1990); Sialyl-Lacto-*N*-neotetraosyl-Cer, Neu5Ac(α2-3)DGal(β1-4)DGlcNAc(β1-3)DGal(β1-4)DGlc-O-R_1, GSC-31 (Kameyama *et al.*, 1990); Sialyl-Sialyl-lactose-Cer, Neu5Ac(α2-9)Neu5Ac-(α2-3)DGal(β1-4)DGlc-O-R_1, GSC-96 (Hasegawa *et al.*, 1992c); Monosialo-ganglioside-GM_1, DGal(β1-3)DGalNAc(β1-4)[Neu5Ac(α2-3)]DGal(β1-4)-DGlc-Cer; Monosialoganglioside-GM_2, DGalNAc(β1-4)[Neu5Ac(α2-3)]-DGal(β1-4)DGlc-Cer; Monosialoganglioside-GM_3, Neu5Ac(α2-3)DGal(β1-4)-DGlc-Cer; Monosialoganglioside-GM_3, Neu5Ac(α2-3)DGal(β1-4)DGlc-O-R_1, GSC-17 (Murase *et al.*, 1989); 8-epi GM_3, GSC-50 (Hasegawa *et al.*, 1992b); 9-deoxy GM_3, GSC-51 (Hasegawa *et al.*, 1992a); Sialyl-lactose-Cer, Neu5Ac(α2-6)DGal(β1-4)DGlc-O-R_1, GSC-61 (Hasegawa *et al.*, 1992d); 4-deoxy GM_3, GSC-75 (Hasegawa *et al.*, 1992a); 7-deoxy GM_3, GSC-76 (Hasegawa *et al.*, 1992a); 8-deoxy GM_3, GSC-77 (Hasegawa *et al.*, 1992a); 4-methoxy GM_3, GSC-78 (Hasegawa *et al.*, 1992b); 9-methoxy GM_3, GSC-79 (Hasegawa *et al.*, 1992b); 4-deoxy-Neu5Ac-lactose, GSC-84 (Hasegawa *et al.*, 1992a).

FACS analysis of trypomastigotes

Monoclonal antibody 3C9 (IgG1) anti-Ssp-3 was purified from ascitic fluids by DEAE–cellulose chromatography. Parasites (2.5×10^6) were washed with 0.2% DMEM–BSA, resuspended in 500 μl of 0.2% DMEM–BSA containing 5 μg/ml of the 3C9 antibody and incubated for 30 min on ice. The suspension was centrifuged, the pellet resuspended in 100 μl of 0.2% DMEM–BSA and 900 μl of 4% paraformaldehyde in PBS added. After 60 min at 4°C, the fixative was removed by centrifugation and the parasites washed twice with 1 ml of cold 0.2% DMEM–BSA. The parasites were resuspended in 500 μl DMEM–BSA and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Boehringer Mannheim) added to a 1/50 dilution. After an incubation of 30 min at 4°C, the suspensions were centrifuged, washed with 0.2% DMEM–BSA, resuspended in 50 μl of PBS and post-fixed with 450 μl of 4% paraformaldehyde. The mixtures were analysed on a Becton Dickinson FACScan. FACS analysis was performed on parasite samples of untreated BSA- and FBS-trypomastigotes, on samples obtained after incubation of these parasites with different saccharides, or after mixing them with each other for 60 min at 37°C.

Cell adhesion assay

L-Cells transfected with E-selectin cDNA were the gift of Dr T.Kishumoto (Boehringer Ingelheim, Ridgefield, CT) and were grown in freshly prepared GPT-selective medium [RPMI 1640 (Gibco BRL, Gaithersburg, MD), 10% FBS, 0.1 mM hypoxanthine, 16 μM thymidine, 250 μg/ml xanthine, 5 μg/ml mycophenolic acid]; parent L-cells were grown in RPMI 1640 supplemented with 10% FBS. Transfected and control cells were plated at a density of 4×10^4 cells/well in 6-well plates (Falcon 3046, Becton Dickinson, NJ) 24 h prior to the assay. Human neutrophils were purified from citrated human blood using Mono-Poly resolving medium (ICN Biomedicals, Flow Laboratories) according to the manufacturer's instructions. The isolated neutrophils were washed three times with DMEM and resuspended in 50 mM DMEM–Pipes (pH 6.5). Cells at 8×10^6 cells/tube were treated with either

0.2 U/ml sialidase (*V.cholerae*, protease-free, azide removed by dialysis, Boehringer Mannheim), purified TS with lactose (1 mM) or an equal volume of PBS. Neutrophils were incubated at 37°C for 90 min, spun down and washed twice with DMEM.

L-Cell cultures were washed once with DMEM without FBS and co-incubated with 2 ml of a suspension of neutrophils at 2×10^4/ml. L-Cells and neutrophils were incubated for 60 min at 37°C on a rotary shaker, after which the medium was removed and the wells washed six times with DMEM without FBS. Rosetting of neutrophils around the adherent L-cells was evaluated microscopically and expressed as percent of L-cells with more than one neutrophil attached.

Acknowledgements

We thank Mr Ching Huang for expert technical assistance. This work was supported by grants from the MacArthur Foundation, the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, Conselho Nacional de Desenvolvimento Cientifico e Tecnologico and Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (Brazil) F.V. is a Research Assistant of the Belgian National Fund for Scientific Research (NFWO), L.P.C is a recipient of a grant from the Secretaria de Ciencia e Tecnologia (Programa RHAE), Brazil.

Abbreviations

BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody, MU-Neu5Ac, 4'-methyl-umbelliferyl *N*-acetyl neuraminic acid; Neu5Ac, *N*-acetyl-neuraminic acid; SA, sialic acid, TS, trans-sialidase

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Received on July 10, 1992; accepted on September 7, 1992