

## Resialylation of sialidase-treated sheep and human erythrocytes by *Trypanosoma cruzi* trans-sialidase: restoration of complement resistance of desialylated sheep erythrocytes

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*Trypanosoma cruzi* trans-sialidase (TS) is a recently described enzyme which transfers  $\alpha(2-3)$ -linked sialic acid from host-derived sialylated glycoconjugates to parasite surface molecules [Schenkman *et al.* (1991) *Cell*, 65, 1117]. We report here on the ability of TS to transfer sialic acid from donor sialyl- $\alpha(2-3)$ lactose to sialidase-treated sheep and human erythrocytes. Up to ~50% resialylation of both desialylated red cells could be attained. Resialylation of desialylated sheep erythrocytes restores their resistance to lysis by human complement. This ascribes a possible biological role for *T. cruzi* TS and demonstrates directly that sialic acid is solely responsible for preventing alternative pathway activation of human complement by sheep erythrocytes.

**Key words:** complement/erythrocytes/sialic acid/sialidase/trans-sialidase

### Introduction

*Trypanosoma cruzi* trypomastigotes express on their surface membranes a unique trans-sialidase (TS) that catalyses the transfer of sialic acid from host glycoconjugates to trypomastigote surface molecules (Schenkman *et al.*, 1991). This enzyme differs from previously described sialyl-transferases since it does not utilize CMP-sialic acid as a donor. Known acceptors for *T. cruzi* TS are saccharides containing terminal  $\beta$ -linked, but not  $\alpha$ -linked galactopyranosyl residues (Vandekerckhove *et al.*, 1992).

Although sialic acid appears to be important for cell invasion by *T. cruzi*, the precise biological role of TS has not yet been defined. Sialic acid is known to be a determinant of complement resistance for some microorganisms (Stevens *et al.*, 1978; Joiner *et al.*, 1984; Mandrell *et al.*, 1990) and it is possible that the sialylation of *T. cruzi* blood stages (trypomastigotes) with host sialic acid serves such a purpose.

The activation of the alternative pathway of complement on cells by bound C3b is influenced by the presence of surface-associated sialic acid residues. Sialic acid enhances the affinity of C3b for serum factor H, a powerful inhibitor of the assembly of the C3 convertase, i.e. C3bBb (Pangburn, 1989; Meri and Pangburn, 1990). Here we utilize this property of sialic acid to assay for the ability of TS to transfer sialic acid to the plasma membrane of cells, and to increase their resistance to complement lysis.

### Results

#### *Sialylation of erythrocytes*

Rabbit, sheep and human erythrocytes were desialylated by treatment with *Vibrio cholerae* sialidase. Table I shows total membrane sialic acid of the erythrocytes before and after sialidase treatment. As expected, very little sialic acid was detected on rabbit erythrocyte membranes (Pangburn *et al.*, 1980), and the amount found on sheep and human erythrocytes is in accord with published data (Pangburn *et al.*, 1980). Sialidase treatment removed >90% of membrane sialic acid from sheep and human erythrocytes. The conditions described for desialylation resulted in maximal release of sialic acid; higher sialidase concentrations or longer incubation times did not result in additional sialic acid release (data not shown).

To determine optimal conditions for the resialylation of erythrocytes, sialidase-treated erythrocytes were incubated with different concentrations of both *T. cruzi* TS and sialic acid donor, sialyl- $\alpha(2-3)$ lactose. Figure 1 shows that increasing the concentration of TS resulted in increased transfer of sialic acid to the surface of sheep and human erythrocytes. In both cells, only ~50% of original surface sialylation was attainable (Table I, columns 1 and 3). Increasing the concentration of sialyl- $\alpha(2-3)$ lactose in the trans-sialylation reaction up to 5 mM (at constant enzyme concentration) also resulted in increased sialylation, but again to a maximum of 50% of initial sialic acid levels (data not shown). The incubation of erythrocytes with either sialyl- $\alpha(2-3)$ lactose or TS alone did not result in any detectable sialylation (not shown). The TS was not able to sialylate rabbit erythrocytes either before or after sialidase treatment.

The amount of sialic acid transferred to erythrocytes under optimal conditions is shown in Table I. The apparent 20% increase in the level of sialylation of untreated sheep erythrocytes by TS (compare columns 1 and 4) is not statistically significant ( $P = 0.277$ ). All erythrocytes used in these experiments were between 2 and 4 weeks old, but storage of erythrocytes for up to 3 months before use did not result in any significant changes in the results obtained.

#### *Sensitivity of erythrocytes to complement*

To demonstrate that resialylation via TS can restore the functional properties of cells with reduced amounts of surface sialic acid, we assayed for their ability to activate the complement cascade. Rabbit erythrocytes, which lack surface sialic acid, are rapidly lysed by the alternative pathway of human complement (Platts-Mills and Ishizaka, 1974) whereas sheep erythrocytes, which bear surface sialic acid, are resistant. Removal of sialic acid from sheep erythrocytes renders them sensitive to human complement (Fearon, 1978; Figure 2).

The effect of resialylation of desialylated sheep erythrocytes by TS was assessed by measuring haemolysis in the presence of human complement. Untreated, desialylated and resialylated sheep erythrocytes were incubated with different concentrations

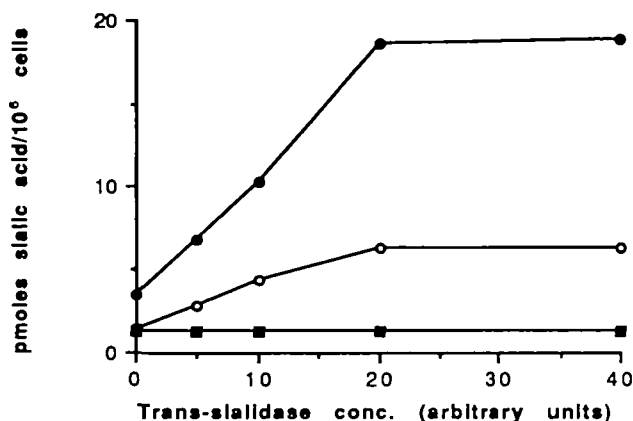
**Table I.** Erythrocyte membrane sialic acid (pmol sialic acid/ $10^6$  cells) before and after treatment with sialidase and TS

Erythrocyte	Untreated	NA <sup>a</sup> only	NA then TS <sup>b</sup>	TS only
Rabbit	1.5 ± 0.13 <sup>c</sup>	1.3 ± 0.31	1.3 ± 0.28	1.6 ± 0.28
Sheep	15.3 ± 2.3	1.4 ± 0.31	6.3 ± 0.57	18.1 ± 3.1
Human	40.8 ± 3.2	3.5 ± 0.61	18.9 ± 1.14	40.0 ± 3.5

<sup>a</sup>NA = sialidase. Figures represent the maximum amount of desialylation attainable.

<sup>b</sup>TS = trans-sialidase. Figures represent the maximum amount of resialylation attainable

<sup>c</sup>Standard deviation ( $n = 3$ ).

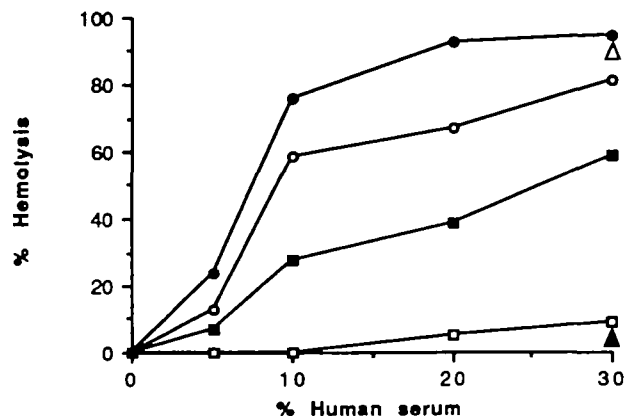


**Fig. 1.** Resialylation of sialidase-treated erythrocytes by TS. Rabbit (■), sheep (○) and human (●) erythrocytes were initially treated with *V. cholerae* sialidase. The desialylated erythrocytes were washed and resuspended to  $1 \times 10^9$ /ml in HBS containing 2 mM sialyl- $\alpha(2-3)$ lactose. An equal volume of *T. cruzi* TS at the indicated concentration was then added and the mixture incubated at 37°C for 60 min. Erythrocytes were thoroughly washed and total membrane sialic acid determined

of human serum at 37°C for 30 min. Figure 2 shows that partial resialylation of sheep erythrocytes resulted in partial restoration of human complement resistance. Depending on the serum concentration, surface resialylation to ~50% of original levels resulted in up to a 50% increase in complement resistance as compared to desialylated erythrocytes. Lower levels of erythrocyte resialylation were less effective in restoring complement resistance, and the level of restoration of complement resistance was approximately proportional to the level of resialylation (Figures 1 and 2). In all cases, haemolysis was due to alternative pathway activation since C2-depleted human serum was as effective as normal human serum, whereas factor B-depleted human serum failed to cause lysis (Figure 2). Nevertheless, TS-treated rabbit erythrocytes remained sensitive to human complement (not shown).

## Discussion

Sheep erythrocytes were converted from non-activators to activators, and back to non-activators, of human complement by sialidase and TS treatment, respectively. This provides an additional direct demonstration of the importance of plasma membrane-associated sialic acid in conferring resistance to lysis by complement. That the lysis was mediated by alternative pathway activation was demonstrated by the use of C2- or factor B-depleted sera, which are defective in the classical or



**Fig. 2.** Haemolysis of untreated desialylated and resialylated sheep erythrocytes. Sheep erythrocytes were desialylated as described, resuspended to  $1 \times 10^9$ /ml in HBS and then incubated at 37°C for 60 min in an equal volume of HBS (●), or *T. cruzi* TS diluted 1/2 (■) or 1/8 (○). Untreated sheep erythrocytes were used as a control (□). Single data points are shown for desialylated erythrocytes incubated in C2-depleted (△) and factor B-depleted (▲) human serum. Following the incubations, erythrocytes were washed thoroughly in PBS and resuspended to  $5 \times 10^8$ /ml in GVB<sup>2+</sup>. For haemolysis,  $5 \times 10^7$  erythrocytes were resuspended in the indicated final dilution of human serum, in a total volume of 200  $\mu$ l. After 30 min at 37°C, 1 ml GVB<sup>2+</sup> was added and haemolysis determined by measuring the absorbance of the supernatants at 412 nm. Complete (100%) lysis was achieved by the addition of H<sub>2</sub>O instead of serum to the erythrocytes.

alternative pathways of complement activation, respectively. As shown elsewhere, desialylated human erythrocytes are not lysed by human complement due to homologous restriction conferred by the membrane proteins DAF (Nicholson-Weller *et al.*, 1982) and CD59 (Lachmann, 1991). The majority of human erythrocyte sialic acid is attached to O-linked oligosaccharides of glycophorin A, 50% being attached by  $\alpha(2-3)$  linkage to a terminal  $\beta$ Gal and 50% being attached by  $\alpha(2-6)$  linkage to an internal  $\alpha$ GalNAc (Anstee, 1981). *Vibrio cholerae* sialidase will remove both types of sialic acid. That *T. cruzi* TS is able to resialylate human erythrocytes only up to ~50% of their original level is in accord with the fact that the TS is only able to link  $\alpha(2-3)$  sialic acid to terminal  $\beta$ -galactopyranosyl residues (Vandekerckhove *et al.*, 1992). A similar explanation would appear likely for the 50% resialylation attainable with sheep erythrocytes. Since the level of protection conferred to sheep erythrocytes is approximately proportional to the amount of sialic acid transferred back to desialylated erythrocytes, it appears that the total amount or surface density of sialic acid is the predominant factor controlling alternative pathway activation. Since the type of sialic acid linkage is known to be important for some biological functions (Tomlinson and Taylor, 1985; Foxall *et al.*, 1992; Kelm *et al.*, 1992; Orlandi *et al.*, 1992), it is possible, but considered unlikely, that  $\alpha(2-3)$ -linked and not  $\alpha(2-6)$ -linked sialic acid is involved in mediating alternative pathway activation. To test this, it would be necessary to correlate complement-mediated haemolysis with  $\alpha(2-6)$  resialylation using a  $\alpha(2-6)$  sialyl-transferase.

Rabbit erythrocytes were not sialylated by *T. cruzi* TS, most likely due to the absence of suitable acceptors. Indeed, unlike human and sheep erythrocytes, rabbit erythrocytes are known to contain abundant terminal  $\alpha$ Gal (Galili *et al.*, 1987).

This is the first report demonstrating the use of exogenously added purified *T. cruzi* TS to sialylate heterologous surface membranes. Our findings also provide evidence that the circulating TS found in patients with acute Chagas' disease

(Affranchino *et al.*, 1989) may alter the structure of cell surfaces and contribute to pathology. The TS may also have some practical applications. Since it can utilize as donors any glycoconjugates bearing sialic acid  $\alpha(2-3)$ -linked to galactopyranosyl residues, TS appears to be more economical than the CMP-sialic acid-dependent  $\alpha(2-3)$  sialyl-transferases for this purpose. In addition, the specificity of the *T. cruzi* enzyme renders it potentially useful in the analysis of sugar composition.

## Materials and methods

### Cells and reagents

Human erythrocytes were isolated from the blood of healthy volunteers drawn into EDTA. Sheep and rabbit erythrocytes were isolated from blood in Alsever solution (Colorado Serum Company, Denver, CO). Erythrocytes were washed four times in phosphate-buffered saline (PBS) just before use. Protease-free *V. cholerae* sialidase was purchased from Boehringer Mannheim (Indianapolis, IN) and TS was purified from the culture supernatant of *T. cruzi*-infected LLC-MK<sub>2</sub> cells by affinity chromatography as described previously (Schenkman *et al.*, 1992). Normal human serum (0.5 U/ml in the above buffer at pH 5.5) were incubated for 60 min at 37°C. Erythrocytes were then washed five times in PBS before resuspension in appropriate buffer for subsequent experiments.

### Desialylation of erythrocytes

Erythrocytes were washed in PBS and resuspended to  $1 \times 10^9$ /ml in 130 mM NaCl, 50 mM Na acetate (pH 6.5) containing 0.01% HSA. Equal volumes of erythrocytes and *V. cholerae* sialidase (0.5 U/ml in the above buffer at pH 5.5) were incubated for 60 min at 37°C. Erythrocytes were then washed five times in PBS before resuspension in appropriate buffer for subsequent experiments.

### Sialylation of erythrocytes

Untreated or sialidase-treated erythrocytes were resuspended at  $1 \times 10^9$ /ml in 150 mM NaCl, 10 mM Hepes (pH 7.0) (HBS) and an equal volume of erythrocytes was mixed with equal volumes of *T. cruzi* TS at different concentrations. Sialyl- $\alpha(2-3)$ lactose was then added to a final concentration of between 0.1 and 5 mM, and the mixture incubated at 37°C for 60 min. TS dilutions were made in HBS. Erythrocytes were then washed five times in PBS before resuspension in appropriate buffer for subsequent experiments. Amounts of TS used are indicated in arbitrary units and represent dilutions of a concentrated stock of affinity purified enzyme (see Schenkman *et al.*, 1992).

### Sialic acid determination

Erythrocytes were lysed and the membranes washed five times in 5 mM Na phosphate buffer (pH 7.8). The membrane pellet was resuspended in an equal volume of phosphate buffer, followed by an equal volume of 0.2 M H<sub>2</sub>SO<sub>4</sub> (0.1 M final concentration). The membranes were then hydrolysed at 80°C for 60 min and the free sialic acid released assayed by an adaptation (Powell and Hart, 1986) of the thiobarbituric acid method (Warren, 1959).

### Complement haemolytic assay

One hundred microlitres of untreated, desialylated or resialylated erythrocytes were resuspended at  $5 \times 10^8$ /ml in GVB<sup>2+</sup> (gelatin veronal buffered saline containing 0.15 mM Ca<sup>2+</sup> and 1 mM Mg<sup>2+</sup>), and mixed with equal volumes of human serum diluted in GVB<sup>2+</sup>. Incubations were carried out for 30 min at 37°C and the reaction stopped by the addition of 1 ml ice-cold GVB<sup>2+</sup>. Following centrifugation, the extent of haemolysis was determined by measuring the absorbance of the supernatants at 412 nm. Complete (100%) lysis was achieved by the addition of H<sub>2</sub>O.

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## Abbreviations

GVB<sup>2+</sup>, gelatin veronal buffered saline containing 0.15 mM Ca<sup>2+</sup> and 1 mM Mg<sup>2+</sup>, PBS, phosphate-buffered saline; TS, trans-sialidase.

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