Sialic Acid in Sickle Cell Disease
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Neuraminic (sialic) acid concentrations in serum from normal and sickle cell (HbSS) subjects were determined for discrete age groups from childhood through adolescence. Values in sickle cell disease were consistently lower over the entire age range. We further investigated the effect of exogenous sialic acid on the rate of sickling reversion of HbSS erythrocytes and demonstrated that this compound in millimole per liter concentrations could revert pre-sickled erythrocytes to their normal morphology in a concentration-dependent manner. When subjected to partial de-sialation with sialidase (EC 3.2.1.18), the HbSS erythrocytes not only sickled faster upon deoxygenation, they also reverted more slowly to treatment with phenylalanine (a more efficient anti-sickling agent than sialic acid) than did untreated cells. We conclude that, in sickle cell disease, erythrocyte sialic acid content could play a significant role, not only in the control of the sickling rate in vivo, but also, after sickling has occurred, in the rate of recovery from a sickling crisis.

Additional Keyphrases: sickling with sodium metabisulphite and reversion · HbSS erythrocytes · pre-sickled cells · neuraminic acid · age-related effects, serum sialic acid

Sickle cell disease (SCD) is a hemoglobinopathy in which the erythrocytes, on deoxygenation, assume bizarre shapes as the hemoglobin crystallizes.1 This crystallization produces the sickling phenomenon, which is the major cause of sickle cell crisis (1). Re-oxygenated, most of the erythrocytes revert to a normal shape, but some of the cells remain irreversibly sickled, implying a possible involvement of the erythrocyte membrane in the sickling process (2, 3). It is also becoming increasingly evident that some abnormal manifestations of sickle cell erythrocytes, such as high accumulation of intracellular calcium ion and deficient ATPase pump, result from a defective erythrocyte membrane (4). Sialic acid, a major component of membrane glycoprotein, is important in maintenance of cellular integrity. Recognition of this has lead in recent years to the investigation of its involvement in several diseases, including SCD and thalassemia (5, 6). These studies have shown that, compared with normal individuals, there is marked decrease in sialic acid content in thalassemia. But not much information is available on the distribution by age on the possible physiological relationship between sialic acid in plasma and SCD. Our objective here was to estimate the plasma content of sialic acid in SCD and to assess its possible role in the etiology of the disease.

Materials and Methods

Blood Collection

Venous blood was sampled from 66 subjects of both sexes, whose homozygous sickle cell condition (HbSS) was confirmed by electrophoresis of the hemoglobin on cellulose acetate. Blood was also collected from 30 normal subjects of HbAA genotype and of both sexes. Both groups (sicklers and non-sicklers) fell within the same age range, 2–28 y, and the consent of all subjects (including the parents of minors) was obtained before blood was collected.

For studies of sialic acid we used serum. For other studies that required uncoagulated blood, we used Na2EDTA as the anticoagulant. Preliminary screening eliminated from our studies those sicklers who had had a blood transfusion not later than three months before this study began. The sicklers were, however, either in crisis or in the steady state at the time they visited our clinics for blood collection. All serum samples were stored at –20 °C until analysis.

Reagents

Commercial kit for sialic acid. This was purchased from Boehringer Mannheim GmbH, Mannheim, F.R.G. The kit was used, with negligible modification, as directed by the suppliers.

Sialidase (EC 3.2.1.18). The lyophilized enzyme (as purchased, unstandardized for specific activity) was also from Boehringer Mannheim. A stock solution of the sialidase was prepared, to give a concentration of 1 mg of lyophilized enzyme per milliliter in Tris acetate buffer, 150 mmol/L (pH 6.5), as adapted from McBride and Rodgerston (7), and 100-μL portions were stored at –20 °C. The Tris buffer was also used as the assay buffer when we determined the sialidase activity of the lyophilized enzyme.

Sialic acid. This was of analytical grade and was purchased from Sigma Chemical Co., St. Louis, MO 63178.

Phosphate-buffered saline (PBS). PBS, 200 mmol of phosphate per liter, pH 7.5, was prepared in distilled water as previously described (8), except that no polyethylene glycol was included.

Buffers stored at 4 °C remained stable for at least two months.

Procedure

Standardization of the sialidase. We diluted our stock sialidase solution (1 g/L in Tris buffer, pH 6.5) with the same buffer to obtain enzyme concentrations ranging from 0.1 to 0.4 mg/mL.

Erythrocytes (HbSS or HbAA) were washed three times with PBS and finally resuspended in the Tris buffer (pH 6.5) to give a packed cell volume of 10% of the suspension volume. We incubated 200 μL of the resuspended cells at 37 °C for 5 min. A final enzyme protein concentration of 5 μg/mL in the assay mixture hydrolyzed, without noticeable hemolysis, some of the sialic acid (assayed with the sialic acid commercial kit). This enabled us to establish the specific activity of the sialidase as that which converted 15.8

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1. Nonstandard abbreviations: SCD, sickle cell disease; HbSS, HbAA, subjects homozygous for sickle cell and for normal hemoglobin, respectively; PBS, phosphate-buffered isotonic saline; Phe, phenylalanine.

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μmol of sialic acid per milligram per minute (i.e., 15.8 kU/g) at 37 °C and pH 6.5.

Assay of sialic acid in serum. The serum samples were batched by age groups, and serum sialic acid was determined for each group with the colorimetric kit, which we also used in determining the sialic acid released from erythrocytes after sialidase action.

Sickling induction of HbSS erythrocytes. We produced rapid sickling of sickle cell erythrocytes by using 20 g/L sodium metabisulphite, as previously described (9). In this method, one volume of undiluted HbSS blood and three volumes of the sodium metabisulphite solution were placed in Luckham LP3 disposable test tubes (63.5 mm × 10.5 mm, purchased from Luckham Ltd., Burgess Hill, Sussex, U.K.) in such a way as to nearly fill up the tube, but allowing a little air space. The tube was capped tightly and made further air-tight by sealing with paraffin wax. Each tube and its contents were mixed gently by rolling for about 1 h, at the end of which at least 45% of the cells had sickled.

Reversion of sickled cells with sialic acid. To estimate the ability of sialic acid to reverse pre-sickled HbSS erythrocytes, we incorporated exogenous sialic acid into the following assay system: We mixed together, in an LP3 disposable 2.5-mL plastic tube, 1.9 mL of blood whose erythrocytes were about 60 to 70% pre-sickled in sodium metabisulphite and 0.1 mL of stock solution of sialic acid prepared with PBS. The tube was tightly capped with its plastic cap, the contents were mixed manually, and a drop of the mixture was quickly withdrawn at various intervals and placed on a microscope slide; the percentage of sickled cells was estimated by counting at least 500 erythrocytes. A control experiment was always included, in which an equivalent volume of PBS replaced the sialic acid.

Sickling rate of sialidase-treated HbSS erythrocytes. We incubated 200 μL of sialidase (3.2 U) and 800 μL of blood (10% packed cell volume) in 150 mmol/L Tris acetate buffer, pH 6.5, at 37 °C in Luckham LP3 disposable plastic tubes for precisely 30 min. We stopped the reaction by adding 2.0 mL of a 20 g/L solution of sodium metabisulphite, which also initiated the sickling of the sialidase-treated erythrocytes. While the tube was being constantly rotated to mix its contents, a drop of the mixture was withdrawn at intervals for microscopic estimation of the percentage of sickled cells. A control experiment was set up in parallel, in which the Tris acetate buffer replaced the enzyme.

Phenylalanine reversion of sickling in sialidase-treated HbSS erythrocytes. We first pre-sickled sialidase-treated erythrocytes with 20 g/L sodium metabisulphite solution as described above. About 1 h of deoxygenation time with the sodium metabisulphite ensured that 50 to 70% of the erythrocytes were sickled.

Sickling reversion of the pre-sickled erythrocytes was achieved by adding, to give a final assay concentration of 5 mmol/L, phenylalanine (Phe), previously shown (10) to be an anti-sickling agent. We have also demonstrated (unpublished) that this amino acid can revert sickled cells to a normal morphology. After Phe was added to the erythrocyte/sodium metabisulphite mixture (pre-sickled erythrocytes), samples were withdrawn at intervals for assessment of the percentage of residual sickled cells. Appropriate control experiments were also set up, in parallel: (a) HbSS erythrocyte/sodium metabisulphite mixture left together for the duration of the experiment without Phe, and (b) non-enzyme-treated HbSS erythrocytes/sodium metabisulphite mixture, to which Phe was added to give the same concentra-

tion. These controls were also sampled at intervals for the estimation of percentage of sickled cells.

At least 10 assay determinations were done for each test and control experiment, permitting a statistical comparison of the mean rate of sickle cell reversion for enzyme-treated and untreated HbSS erythrocytes with Phe, which is a better anti-sickling agent than sialic acid.

Statistical analysis of results. The mean and SD for the sialic acid values at different age groups were calculated for the sicklers and non-sicklers. The mean rates of sickled cell reversion for enzyme-treated and untreated cells were also computed. The significance of the results was estimated by using Student’s t-test (11).

Results

Figure 1 shows sialic acid values for both normal and sickle cell serum samples. In comparison with normal subjects, lower concentrations of sialic acid were observed in the sickle cell samples from all the age groups. Except for the age group 0 to 5 y, where the difference was not significant (P > 0.2), the difference was highly significant (0.05 < P > 0.01). Additionally, there was a progressive increase in serum sialic acid with age, rapidly initially but leveling out from about the age of 15 y upwards (Figure 1). Although information regarding the sialic acid content of erythrocyte membrane in SCD is available in the literature (5, 6), we could not find similar information for serum or plasma, especially with regard to its variation with age, in this disease.

Effect of sialic acid on sickling. Exogenous sialic acid was shown to reverse pre-sickled erythrocytes (Figure 2), and this process was shown to be concentration dependent. For example, we observed that sialic acid at 8 mmol/L reversed about 50% of the sickled cells to their normal shape within 15 min in vitro, whereas at a 2 mmol/L concentration, only about 10% of sickled cells reverted during about the same interval.

The effect of sialidase treatment on sickling rate. Figure 3 shows the sickling rates of enzyme-treated and untreated HbSS erythrocytes. It is evident that the sickling rate was consistently and significantly slower in the untreated (control) erythrocytes. For example, we found that the initial
sickling rate in 20 min was about 20% (± 3%) and 13% (± 4%) for enzyme-treated and untreated cells, respectively. This difference in sickling rate is highly significant (P < 0.01).

**Sickling reversion of sialidase-treated erythrocytes.** Figure 4 shows, relative to the control, Phe-induced sickle cell reversion of sialidase-treated and untreated HbSS erythrocytes. We observed that de-sialation of these erythrocytes resulted in cells that less readily reverted to normal morphology after they had been pre-sickled. The percentage of reversion of sickled cells was about 20% in 50 min and 20% in 25 min for treated and untreated cells, respectively, implying a twofold slowing in the rate of reversion for the enzyme-treated erythrocytes.

**Discussion**

**Serum sialic acid.** There is a significant decrease in membrane sialic acid in SCD (5, 6). Our study has shown that a similar decrease in sialic acid occurs in the serum of sicklers, possibly implying that the loss of sialic acid in erythrocytic membrane is reflected in a commensurate removal of the compound from the circulation. It is unclear why the decrease in sialic acid in plasma (as well as in membrane) is age-dependent in SCD, but proteolytic changes do occur in mammalian erythrocytes at various stages of maturation (12).

We observed the least sialic acid changes between normals and sicklers at ages 0 to 5 y. SCD does not manifest very early in life because of certain factors such as the generally high concentration of fetal hemoglobin in the young (13). It is, however, uncertain whether the near-normal sialic acid in this age range plays any stabilizing role in sickle cell crisis.

**Sialic acid and the sickling phenomenon.** Sialic acids are basically charged sugars of various molecular sizes and complexity. Perhaps they exert their anti-sickling role in a manner similar to that reported for other sugars (14). The sialic acid we used in this work is a fairly small molecule (Mr 309). Possibly it exerts its anti-sickling action by gaining access into the erythrocyte. Other modes of action are also possible, such as interacting with the membrane to effect an internal conformational change favorable to the anti-sickling process. Interestingly, the mean serum sialic acid concentrations in all ages we studied fell just outside the effective anti-sickling threshold of about 3 to 4 mmol/L observed in vitro. Therefore, plasma sialic acid in SCD would be expected to contribute little to the anti-sickling process in this disease. Clearly, in molar terms, sialic acid is far less effective as an anti-sickling agent than is Phe or uric acid (15). Its smaller concentration in SCD is certainly a disadvantage to the patient.

Sialic acid-deficient HbSS erythrocytes, which we produced by partly digesting them with sialidase, sickled more rapidly, but reverted more slowly, than untreated cells. In vitro, we were then able to mimic the effect of irreversible sickle cells. Information is not readily available in the literature about sialic acid concentrations in erythrocyte membranes in reversible and irreversible sickle cells. However, cells with membranes that are more deficient in sialic acid should be expected to be removed faster from circulation by the reticuloendothelial system, e.g., by the spleen. Such cells include HbSS erythrocytes. The slow reversion rate of sialidase-treated HbSS erythrocytes suggests that the rate of in vivo sickling phenomenon may in fact be contributed to by the sialic acid content of the erythrocyte membrane. This may indeed be so, because sialic acid-
deficient erythrocytes should be expected to repel one another less, clump together more, and compete more avidly for the already decreased oxygen in the microvasculature.

The slow reversion rate of sialic acid-deficient HbSS erythrocytes, when challenged with a sickling reversal agent such as Phe, should be considered an important factor in the therapeutic management of SCD. If this in vitro phenomenon can be reproduced in vivo, it could imply that erythrocyte sialic acid content in SCD would play an important role in the rate of recovery from a sickling crisis, as well as in the severity of such crisis.

We observed a higher (by about 50%) sialic acid concentration in serum from the non-sickler, especially in adulthood, when compared with some values in the literature. The reason for this difference in value is unclear. However, it is well documented that sialic acid concentrations are increased in various pathological conditions, including inflammations and general infections (16, 17). No definite conclusion based on pathology can be drawn for the Nigerian populace until sialic acid values in serum from discrete age groups have been measured in a much larger population.

References