Inhibition of Sickling in Erythrocytes by Amino Acids

By N. M. Rumen

Sickling of erythrocytes was rapidly and quantitatively inhibited at room temperature by 3.8 mM homoserine, asparagine, and glutamine but by no other amino acid.

PREVIOUS ATTEMPTS to inhibit sickling of human erythrocytes containing hemoglobin S have been made, using urea at high concentration,[1] cyanate,[2] and carbamyl phosphate.[3] However, these reagents exhibit varying degrees of toxicity and are relatively unsuitable for clinical use. It seemed, therefore, of particular interest that specifically three amino acids, L-homoserine, L-asparagine, and L-glutamine decreased the CO-binding affinity of hemoglobin S, while no effect of these or any other amino acids could be demonstrated with hemoglobin A.[4] This observation raised the possibility that these amino acids might inhibit sickling of erythrocytes in sickle cell disease. Evidence to support this hypothesis is presented in this report.

METHODS

Preparation of Red Cells

Red cells containing hemoglobin S were collected 2–4 hr prior to use (courtesy of Dr. P. McCurdy of D. C. General Hospital; Dr. P. Jilley of Freedman's Hospital; Dr. H. H. Waxman of Temple University Medical School, Philadelphia, Department of Hematology and Dr. T. Schwarz of Albert Einstein Medical School, Philadelphia, Pa.). They were suspended in a volume of 0.9% NaCl threefold that of the packed cells, and were either used directly or washed with normal saline, centrifuged at 12,000 *g* (Sorvall Refrigerated Centrifuge) at 4°C for 10 min, then resuspended and centrifuged as above an additional three times.

Deoxygenation and Microscopic Analysis

An aliquot of approximately 50 µl of the suspended erythrocytes was spread on a microscope slide and mixed at room temperature with 15 µl of a 0.02 *M* solution of one of the three aminoacids for a total period of 5 min, leading to a final aminoacid concentration of approximately 3.8 m*M*. The suspension was deoxygenated by addition of 15 µl 2% sodium-dithionite in 0.9% NaCl prepared within 15 min of use; it was rapidly covered with a glass coverslip and the edges of the coverslip were sealed with vaseline or dental wax. Cell suspensions containing higher concentrations (0.3 *M*) of sodium chloride and excess of dithionite were alternatively employed.

In an alternative deoxygenation method, 2–3 ml of the cell suspension and amino acid solution to a final concentration of 70 m*M* were placed into a two-way stopcock tonometer provided with a sidearm sealed by rubber stopper. The cells were deoxygenated for 45 min by a flow of water-saturated nitrogen through the tonometer. Aliquots were withdrawn by syringe through the rubber stopper, and placed on a microscope slide. The slide was immediately placed into a glovebox filled with nitrogen and was sealed. Control experiments were carried out in the absence of amino acids.

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Freshly prepared slides were inspected and photographed (Zeiss No. 61499 microscope with 5-600-fold magnification equipped with a Polaroid MP-3 or 35 mm camera). Cell counts were made by means of a grid either directly under the microscope or from the photographs.

Amino Acids

L-homoserine, L-glutamine, and L-asparagine (A Grade) were obtained from Calbiochem.

RESULTS

Figure 1 A-1D shows that the reaction of asparagine, glutamine, and homoserine with erythrocytes containing hemoglobin S leads to a high degree of inhibition of sickling.

Cells suspended in 0.3 *M* sodium chloride and deoxygenated with an excess

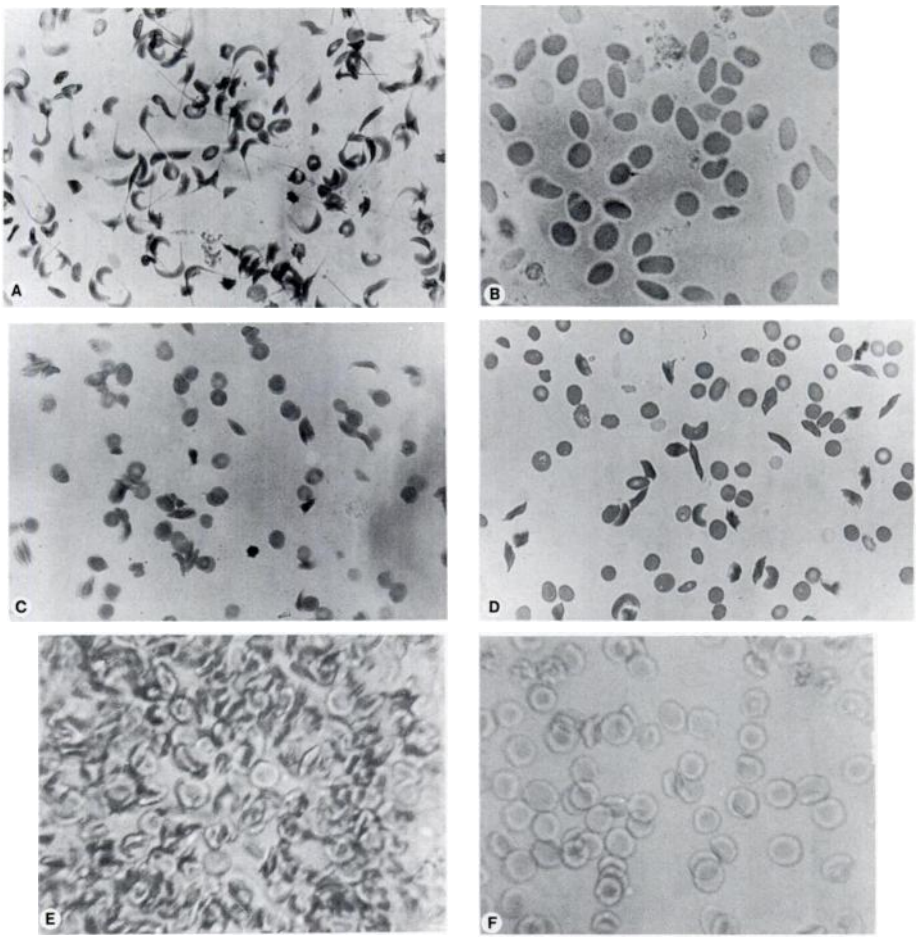

Fig. 1. Microscopic view of (A) deoxygenated sickled erythrocytes, (B) deoxygenated sickled erythrocytes, 3.8 mM in asparagine, (C) deoxygenated sickled erythrocytes, 3.8 mM in glutamine, (D) deoxygenated sickled erythrocytes, 3.8 mM in homoserine, (E) deoxygenated (hemoglobin S-C) erythrocytes, (F) deoxygenated (hemoglobin S-C) erythrocytes, 3.8 mM in homoserine.

Table 1

Amino Acid Added to Sickled Cells	Sickled Cells %	CV*	n†
None	92.5	2.5	10
Homoserine	12.4	9.4	7
Glutamine	9.4	8.8	7
Asparagine	11.3	8.5	7

*Coefficient of variation.
†Number of samples.

of dithionite showed an increased degree of sickling in the absence of amino acids compared to that in 0.15 *M* NaCl and 2% sodium dithionite.

Inhibition of sickling by amino acids was the same whether deoxygenation was carried out by dithionite or by nitrogen. The deoxygenated cells were preserved at 4°C for periods of at least 4–5 wk. However, after 4–5 days, sickling progressively reappeared. It could be repeatedly inhibited by successive additions of amino acid solutions.

Table 1 describes the degree of sickling inhibition produced by the three amino acids. In all of ten patients studied, sickling was inhibited to at least 90% by each of the three amino acids. Cells from three of ten patients studied exhibited almost quantitative inhibition after addition of homoserine. Five showed complete remission after reacting with glutamine. In a single case, asparagine showed the highest inhibition of sickling. Thus, it seems that the red cells from different patients may respond differently to the three amino acids tested. This phenomenon remains unexplained to date.

Five patients' cells were analyzed five or more times during 1 yr. In each case, the specificity of the response for any particular amino acid remained the same.

Erythrocytes containing hemoglobin SC were allowed to react with the three amino acids. Sickling of these cells was quantitatively inhibited by homoserine, and to a somewhat lesser degree with the other two amino acids (Fig. 1E and 1F).

DISCUSSION

On the basis of flash photolysis experiments, it was found previously[4] that the decrease in carbon monoxide affinity of hemoglobin S by asparagine, glutamine, and homoserine was independent of the presence or absence of 2,3-diphosphoglycerate (DPG), indicating that these amino acids and DPG do not compete for the same binding site. The dissociation between the binding of amino acids and DPG to hemoglobin S contrasts with the close correlation between cyanate and DPG binding to hemoglobin S. Cyanate binds to the α- and β-chains of oxyhemoglobin to about the same extent.[5-7] Yet, cyanate binding to α-chains in deoxyhemoglobin is twice as high as to β-chains.[8] The N-terminal of the β-chain of deoxyhemoglobin is involved in the reversible binding of DPG.[9,10] However, DPG inhibits β-chain carbamylation selectively,[11] which occurs at the identical site, viz., at the N-terminal of the β-chain.[6] Cyanate displaces DPG from the binding site, thereby increasing oxygen affinity,[9] while binding of the specific amino acids decreases it. But this decrease of oxyhemoglobin S may be physiologically harmless, since the same amino acids

also prevent the aggregation of hemoglobin S^{12} and the associated loss of oxygen-carrying capacity.

The efficacy of low concentrations of neutral amino acids in inhibiting the sickling of human erythrocytes is of the greatest clinical promise. It suggests that it might be possible to prevent sickle cell crises by dietary means, and possibly even to provide the symptomatic relief of established crisis.

The mechanism of reaction of the three amino acids with erythrocytes containing hemoglobin S remains unknown. Studies are in progress to elucidate some aspects of this phenomenon by use of gel electrophoresis, solubility assays, and measurement of the rate of hemoglobin gelation and of the oxygen saturation of hemoglobin S.

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