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INHIBITION OF IN VITRO SICKLING BY LIPOSOME-MEDIATED TRANSPORT OF AMINO ACIDS INTO INTACT HUMAN RED BLOOD CELLS*

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To investigate the role of phenylalanine and tryptophane as potential antisickling agents in intact human SS-red blood cells a liposomal transport system was employed to transfer phenylalanine or tryptophane into intact SS-red blood cells. Aromatic amino acids and short peptides containing phenylalanine have been demonstrated to increase the minimum gelling concentration and solubility of deoxy-hemoglobin S in aqueous solution. However, these compounds do not cross the red blood cell membrane under usual incubation conditions. Incorporation of phenylalanine or tryptophane into intact SS-red blood cells via liposomal transport system markedly inhibited the in vitro sickling of deoxy-hemoglobin S. These findings raise the possibility that a nontoxic liposomal transport system which facilitates incorporation of antisickling agents into intact SS-RBC may have significant therapeutic implications in the treatment of sickle cell disease.

INTRODUCTION

Various approaches have been used in attempts to develop antisickling agents for clinical use that will inhibit gelation of deoxy-hemoglobin S and/or stabilize the normal biconcave configuration of the red cell. Recently, chaotropic agents have been employed to disrupt the hydrophobic bonding responsible for the polymerization of Hb S. The most promising chaotropic agents to date appear to be neutral amino acids containing a phenyl ring (1-3) and tri- or tetrapeptides containing phenylalanine (4, 5).

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Abbreviations used are : RBC, red blood cells: Hb, hemoglobin; Phe, phenylalanine: Trp, tryptophane.

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These agents bind to the surface of Hb S molecule and prevent the intermolecular contacts necessary for polymerization. Most of the studies using chaotropic agents to date have employed Hb S in aqueous solution (1, 2) because aromatic amino acids and peptides containing Phe do not enter the intact red blood cell under the usual conditions of incubation.

In order to investigate the role of Phe and Trp as potential antisickling agents in intact human RBC, a liposomal transport system was employed to transfer Phe or Trp into intact human SS-RBC. The effect of the incorporation of Phe or Trp on *in vitro* sickling was then investigated.

METHODS

Preparation of intact SS-RBC. Venous blood from patients with sickle cell anemia was collected in Vacutainer tubes using citrate-phosphate-dextrose as anticoagulant. The blood was centrifuged at 1600 g for four minutes and the plasma and buffy coat were discarded. The packed RBC were then washed twice with three volumes of isotonic phosphate buffer (2.839 g $NaH_2PO_4.2H_2O$, 16.308 g $Na_2HPO_4.2H_2O$ made to one liter with distilled water, pH 7.45. The RBC were then brought to 37°C. For the reversal of sickling experiments, the RBC were deoxygenated for 15-20 minutes until the pO_2 approximates to zero.

Preparation of amino acid loaded liposomes. Liposomes were prepared from a mixture of synthetic phosphatidyl choline (Sigma Chemical Company, St. Louis, MO, USA; catalogue # 4139) and lysophosphatidyl choline (also from Sigma; catalogue # 4129). The lipids in a 1:1 molar ratio, were dissolved in 9:1 $CHCl_3$:CH_3OH mixture and rotary evaporated *in vacuo*. The resulting thin film was resuspended in 0.15 M Phe or 0.05 M Trp, pH 7.4, and sonicated at 30°C under nitrogen for 45 minutes to form liposomes containing either Phe or Trp. After sonication, the liposomes were aliquoted into plastic screw top vials and stored at -20°C.

Incorporation of Phe or Trp into intact SS-RBC *via* Phe or Trp loaded liposomes. Liposome suspensions were thawed and brought to 37°C. Equal volumes, of SS-RBC, and liposome suspension loaded with either Phe or Trp were mixed and the mixture incubated at 37°C for 30 or 60 minutes respectively. After incubation, the RBC suspensions were centrifuged at 400 g for 2-3 minutes, the supernatents were removed and the RBC washed twice with three volumes of isotonic phosphate buffer. The RBC were then resuspended in the same buffer to a final hematocrit of 45 % and aliquots were obtained for the determination of amino acid incorporation.

Liposome incorporated RBC were hemolysed with N/70 sulphuric acid and proteins were precipitated with 10 % sodium tungstate. The supernatents were analyzed for amino acid nitrogen content by the colorimetric method of Folin (6) as modified by Frame (7) and Kachmar (8). The phenylalanine content of the supernatent was also measured by a fluorimetric method described in Sigma Technical Bulletein No. 60-F.

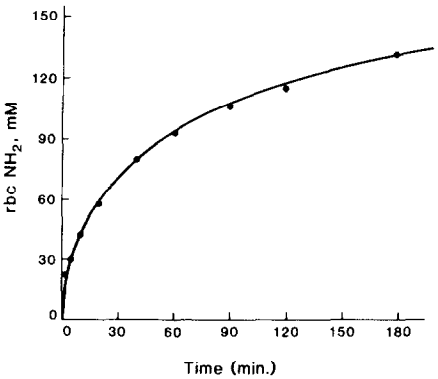

Figure 1. Kinetics of liposome-mediated transport of phenylalanine into intact SS-RBC. T 37°C; pH 7.45 isotonic phosphate buffer.

Kinetics of phenylalanine incorporation into intact SS-RBC. Two milliliter of SS-RBC and two milliliter of thawed liposome suspension containing Phe were mixed and the mixture incubated at 37°C. Aliquots (0.4 ml), were removed and placed in ice at zero time and at 5, 10, 20, 30, 60, 90 and 120 minute time intervals and centrifuged immediately at 10°C. The supernatents were removed and the RBC washed and resuspended in phosphate buffer as described above. Each sample was then analyzed for its amino acid content.

. Inhibition and reversal of in vitro sickling. One milliliter aliquots of SS-RBC samples were equilibrated in an atmosphere of 95 % nitrogen and 5 % carbondioxide until the pO_2 approximates to zero. Twenty-five microliter of the deoxygenated SS-RBC were transfered to one milliliter of deoxygenated phosphate-buffered-gluteraldehyde for fixation. For comparison, liposomes alone were prepared in phosphate buffer and incorporated into SS-RBC and fixed. Also SS-RBC were incubated with 0.15 M Phe for 60 minutes and fixed as above. Red cell morphology was then examined with a Zeiss microscope equipped with differential interference contrast optics.

RESULTS

Figure 1 shows the kinetics of liposome-mediated transport of phenylalanine into intact SS-RBC at 37°C and pH 7.45. Under these conditions, the reaction is characterized by a half-time of 25 minutes; As can be seen from Figure 1 an intracellular concentration of 60 mM Phe was achieved after 30 minutes of incubation. The incorporation of Trp into SS-RBC was however slower and an intracellular concentration of 40 mM Trp achievable after 60 minutes of incubation. Figure 2 illustrates the inhibitory effect of Phe at an intracellular concentration of 60 mM and Trp at an intracellular concentration of 40 mM in preventing the sickling of intact deoxygenated SS-RBC.

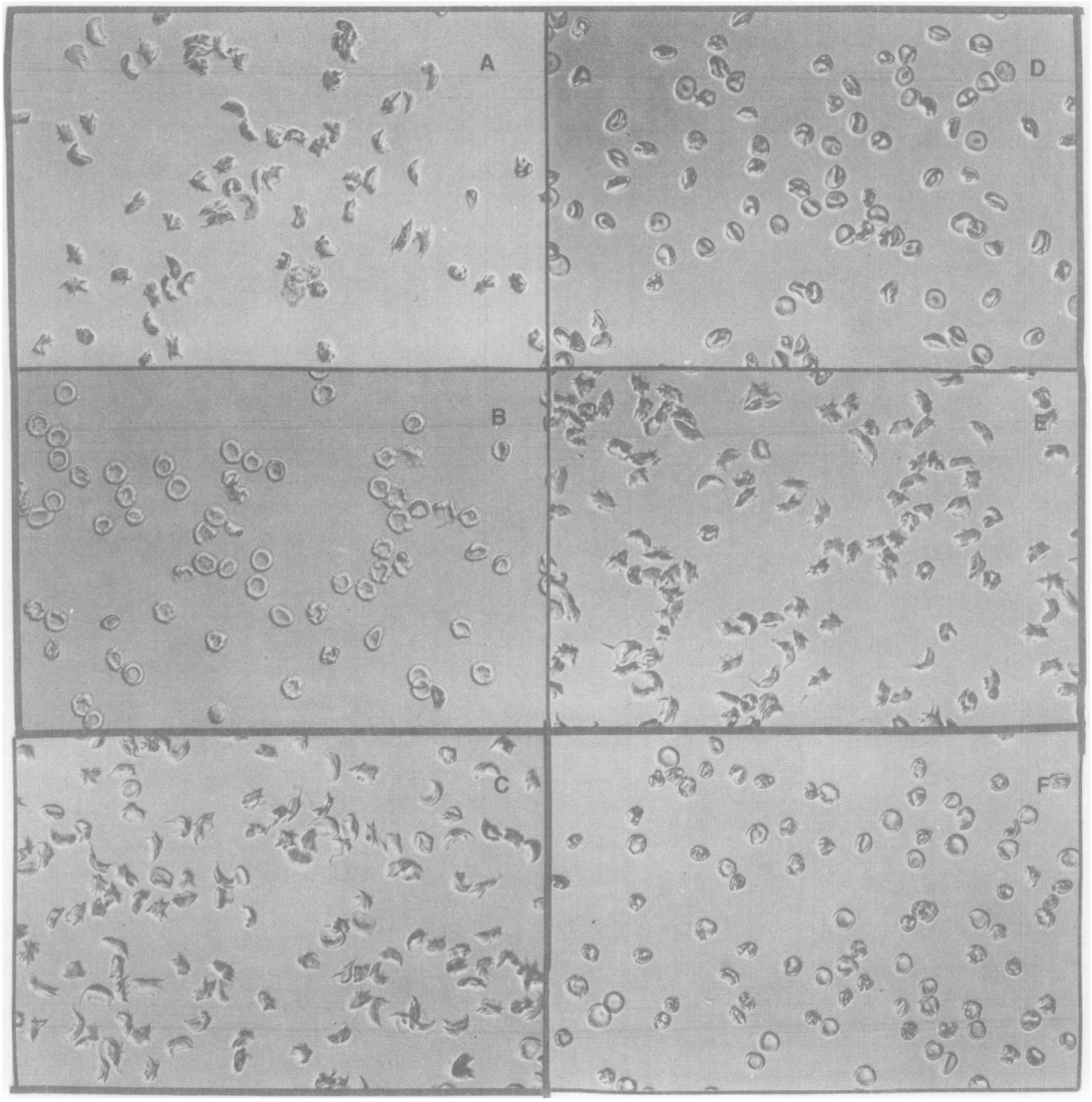

Figure 2. Inhibition and reversal of in vitro sickling of deoxygenated SS-RBC.
A. Untreated SS-RBC (control); B. 60 mM phenylalanine incorporated SS-RBC (sickling inhibited); C. Liposomes alone incorporated SS-RBC; D. phenylalanine incorporated deoxy-SS-RBC (sickling reversed); E. 0.15 M phenylalanine treated SS-RBC; and F. 40 mM tryptophane incorporated SS-RBC (sickling inhibited).
Nomarski Interference Optics : magnification x 300

DISCUSSION

Neutral aromatic amino acids such as Phe and Trp and short peptides containing Phe inhibit polymerization of deoxy-Hb S in aqueous solution (1-3, 9). Permeability studies with ^{14}C-labelled

peptides showed that these compounds do not enter the intact RBC under usual conditions of incubation (4). However, the author has developed a liposomal transport system which transfers aromatic amino acids effectively across the intact RBC membrane.

The substitution of valine for glutamic acid at position six of the β chains of normal human adult hemoglobin is responsible for the polymerization of deoxy-Hb molecules in sickle cell disease (10,11). The mechamism by which these aromatic amino acids destabilize Hb S formation and thereby inhibit sickling of SS erythrocytes remains unknown. Beta^{6} valine is in close intermolecular contact with β-Phe^{85} in the deoxy Hb S crystal (12). This raises the possibility that the inhibitory effect of exogeneously added phenylalanine could be stereospecific. On the other hand, a number of specific Hb S - Hb S interactions could be responsible for the stabilization of microtubular assembly (13). Some of these have strong hydrophobic interactions such as contacts near Phe^{88} or Leu^{88} in the β chain, which are possible sites for the interaction with Val^{6} of the β chain of an adjoining molecule (13).

The present findings indicate that Phe at an intracellular concentration of 60 mM or Trp at an intracellular concentration of 40 mM, when incorporated into intact oxy-SS-RBC and deoxy-SS-RBC inhibit and reverse, respectively, in vitro sickling. Should these effects obtain in vivo the use of potentially nontoxic liposome incorporated phenylalanine or tryptophane could hold great promise in the treatment of sickle cell disease.

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