

A Multiparameter Analysis of Sickle Erythrocytes in Patients Undergoing Hydroxyurea Therapy

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During 24 weeks of hydroxyurea treatment, we monitored red blood cell (RBC) parameters in three patients with sickle cell disease, including F-cell and F-reticulocyte profiles, distributions of delay times for intracellular polymerization, sickle erythrocyte adherence to human umbilical vein endothelial cells in a laminar flow chamber, RBC phthalate density profiles, mean corpuscular hemoglobin concentration and cation content, reticulocyte mean corpuscular hemoglobin concentration, ¹H-nuclear magnetic resonance transverse relaxation rates of packed RBCs, and plasma membrane lateral and rotational mobilities of band 3 and glycophorins. Hydroxyurea increases the fraction of cells with sufficiently long delay times to escape the microcirculation before polymerization begins. Furthermore, high pre-treatment adherence to human umbilical vein endothelial

cells of sickle RBCs decreased to normal after only 2 weeks of hydroxyurea treatment, preceding the increase in fetal hemoglobin levels. The lower adhesion of sickle RBCs to endothelium would facilitate escape from the microcirculation before polymerization begins. Hydroxyurea shifted several biochemical and biophysical parameters of sickle erythrocytes toward values observed with hemoglobin SC disease, suggesting that hydroxyurea moderates sickle cell disease toward the milder, but still clinically significant, hemoglobin SC disease. The 50% reduction in sickle crises documented in the Multicenter Study of Hydroxyurea in Sickle Cell Disease is consistent with this degree of erythrocyte improvement.

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SICKLE CELL DISEASE produces recurrent, severe episodes of pain and extensive organ damage caused by occlusion of the microcirculation, with secondary tissue ischemia.¹ Erythrocytes containing polymerized hemoglobin (Hb) are rigid and occlude the microcirculation. The marked variability in the clinical course of sickle cell disease indicates that factors other than the sickle mutation contribute to the phenotypic expression of the sickle cell genotype.² Fetal Hb (HbF) production is the best characterized of these secondary phenomena. HbF distribution is inhomogeneous, producing two distinct populations of erythrocytes with respect to HbF content. F cells contain about 20% HbF and 80% HbS, whereas "S cells" contain only HbS.^{3,4} Patients with a large fraction of F cells, as occurs in Saudi Arabians, tend to have milder disease.^{5,6} Early gelation studies^{7,8} indicated that decreased Hb polymerization causes the milder disease. The mechanism of this inhibition and its relation to the pathophysiology is now well understood and is surprisingly simple.⁹ The γ subunits of both the $\alpha_2\gamma_2$ homotetramer and the $\alpha_2\beta\gamma$ hybrid tetramer⁹ are largely or completely excluded from the polymer.^{10,11} The concentration of the polymerizing tetramer, $\alpha_2\beta_2$, is consequently lowered by about 35% in a typical F cell. The delay time increases by approximately 1,000-fold, predicting that the vast majority of F cells will pass through the microcirculation before Hb polymerization (unless they are caught in a "log-jam").¹⁰

These clinical and biophysical findings sparked considerable interest in finding ways to stimulate HbF synthesis as a treatment for sickle cell disease. Hydroxyurea is the most successful drug so far. After the initial observations of Platt et al,¹² several studies involving hydroxyurea therapy for small groups of patients¹³⁻¹⁶ showed an increase in HbF levels resulting from an increase in the fraction of F cells.¹⁷ Other notable changes include less erythrocyte dehydration, fewer irreversibly sickled cells, and longer erythrocyte life spans.¹⁸ Patients in these studies generally appeared to have fewer painful crises.¹⁹ This conclusion was dramatically confirmed in the randomized, double-blind "Multicenter Study of Hydroxyurea in Patients with Sickle Cell Disease."²⁰

Understanding how hydroxyurea ameliorates the clinical

condition of patients with sickle cell disease is crucial to advance beyond this first step in the treatment of the disorder. Is clinical improvement due solely to changes in F-cell production, or does hydroxyurea alter other parameters that contribute to vaso-occlusion? To address this question, we assessed a number of erythrocyte biochemical and biophysical parameters in three patients started on hydroxyurea therapy. These include the fraction of F cells, the distribution of delay times, erythrocyte adherence to endothelial cells, the fraction of dense cells, ¹H-nuclear magnetic resonance (NMR) transverse relaxation rates, ion transport properties, and the lateral and rotational mobility of membrane-spanning proteins.

MATERIALS AND METHODS

Patient characteristics. Informed consent for the study was obtained from each patient, consistent with the policies of the Brigham and Women's Hospital (Boston, MA). Patient A was a 36-year-old

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man, patient B was a 26-year-old woman, and patient C was a 33-year-old man. All three patients had homozygous sickle cell disease, proven by Hb electrophoresis. Pretreatment hematologic characteristics (normal mean corpuscular volume, normal mean corpuscular Hb, and mean corpuscular Hb concentration) argued against 2-gene α -thalassemia. Each had a history of severe, recurrent vaso-occlusive sickle cell pain that required three or more hospitalizations per year. No patient had a history of stroke or acute chest syndrome. No patient was treated previously with hydroxyurea or other antisickling agents. No patient had received chronic transfusion therapy.

Patients were started on hydroxyurea at a dose of 20 mg/kg. No patient required a dose adjustment over the course of the study, and none required phlebotomy. No child was conceived by any patient during the study, consistent with the use of effective birth control measures. Patient samples were drawn simultaneously for all experimental measurements at each time point. Samples from different volunteers were used as controls for the different time points.

F-cell and F-reticulocyte measurements. Immunostaining of cells was performed to assess the HbF-containing fractions of total red blood cells (RBCs) and reticulocytes.²¹ Measurements were obtained for each patient twice before hydroxyurea treatment and at approximately 4, 12, and 24 weeks into treatment.

Endothelial cells. Human umbilical vein endothelial cells were harvested from two to five umbilical cord veins, pooled, and grown in primary culture as previously described.²² Cultures were serially passaged (1:3 split ratios) with M199-20% fetal calf serum supplemented with 50 to 100 mg/mL endothelial cell growth factor and 50 to 100 mg/mL porcine intestinal heparin in Costar (Cambridge, MA) tissue-culture flasks coated with 1 mg/cm² purified fibronectin. Experiments were performed with cells at passage no. 2 that were grown to confluence on fibronectin-coated LabTek slides (LabTek, Naperville, IL).

Erythrocyte-endothelial adherence assay. Erythrocyte adherence was quantitated under postcapillary venule shear stress conditions (1 dyne/cm²) using a parallel plate flow chamber as previously described.²³ Endothelial cell-coated slides were assembled in the flow chamber and rinsed for 2 minutes with HEPES-buffered saline solution containing 1% albumin (HBSS/A). Washed RBCs were suspended at 1% hematocrit in HBSS/A and perfused over endothelial monolayers for 10 minutes. Nonadherent RBCs were removed by a 10-minute rinse with cell-free medium. The number of adherent RBCs were counted in 24 random microscopic fields scanned using an inverted phase-contrast microscope and video camera. The average number of adherent RBCs for the 24 fields examined was normalized per square millimeter of endothelium.

¹H-NMR transverse relaxation rate measurement. Packed RBC samples were prepared from freshly drawn venous blood and washed 3 times in a buffered solution (140 mmol/L NaCl, 10 mmol/L TRIZMA base [pH, 7.4]) after removal of plasma and leukocytes. Pellets were transferred to 10-mm-diameter NMR tubes. Transverse relaxation measurements were made at 10 MHz on an IBM Minispec (IBM, Armonk, NY) with a probe temperature of 40°C. A Carr-Purcell-Meiboom-Gill (CPMG) sequence with a 4.8-millisecond echo spacing and even echo sampling of 1,000 echoes was used for data collection. Resulting decay curves were fit with monoexponential decay functions to extract relaxation rates R2. Relaxation rate measurements reflect the content and mobility of water as well as its interaction with intracellular components of the RBC, such as Hb.²⁴⁻²⁶

RBC ion content, density, and ion transport. RBC and reticulocyte indices were measured with the Bayer H*3 Hematology analyzer (Bayer, Tarrytown, NY).²⁷ RBCs were washed 4 times with choline washing solution (144 mmol/L Choline Cl, 1 mmol/L MgCl₂, 10 mmol/L Tris-3-[N-morpholino]propanesulfonic acid [MOPS; pH, 7.40] at 4°C) for measurements of internal Na⁺ and

K⁺ content. RBC density was measured by phthalate density profile. Values of median density (D₅₀) and middle density range (R₄₀) were obtained as detailed.²⁸ The percentage of cells with density higher than 1.112 was also calculated to estimate changes in dense, dehydrated sickle (SS) cells.

K-Cl cotransport. Cotransport from fresh cells was measured as either chloride-dependent or volume-dependent K⁺ efflux. Flux media for chloride-dependent K⁺ efflux contained 100 mmol/L Na⁺ and 1 mmol/L Mg²⁺ (the anion being either Cl⁻ or NO₃⁻), 10 mmol/L glucose, 10 mmol/L Tris-MOPS (pH, 7.4 at 37°C), 0.1 mmol/L ouabain, and 0.01 mmol/L bumetanide. Chloride-dependent K⁺ efflux was calculated as the difference between K⁺ efflux in chloride and nitrate media. Swelling-induced K⁺ flux was calculated as the difference between K⁺ efflux in NaCl hypotonic (100 mmol/L) and in NaCl isotonic (140 mmol/L) media. Incubation times at 37°C for flux measurements were 5 and 15 minutes.¹⁸

Ca²⁺-activated ⁸⁶Rb influx. The activity of the Ca²⁺-activated K⁺ channel (Gardos Channel) was estimated by measuring the Ca²⁺-activated ⁸⁶Rb efflux.²⁹

Lateral and rotational mobility. Fresh blood was collected by venipuncture into heparinized tubes. The buffy coat was immediately removed by aspiration, and RBCs were washed 3 times and stored at 4°C in 140 mmol/L KCl, 15 mmol/L NaPO₄, 10 mmol/L glucose (pH, 7.4). RBC band 3 and glycophorins were fluorescently labeled with eosin-5-maleimide and fluorescein-5-thiosemicarbazide, respectively, as described.³⁰ Discontinuous Stractan density gradients were used to separate fluorescently labeled cells into six different density fractions, ranging from 1.081 to 1.160, as described.^{31,32} Cells from the 1.081 to 1.085 (low density), 1.094 to 1.107 (medium density), and 1.111 to 1.160 (high density) g/mL interfaces were used. These fractions had mean cell Hb concentrations of 30, 37, and 42 g/dL.³⁰

Fluorescence photobleaching recovery was used to measure the lateral mobility of fluorescently labeled band 3 and glycophorins, as described.³⁰ Briefly, a Gaussian laser beam was focused to a spot on a fluorescently labeled RBC in a fluorescence microscope. After an intense photobleaching pulse, recovery of fluorescence was monitored by periodic low-intensity pulses. Recovery resulted from the lateral diffusion of unbleached fluorophores into the bleached area. Nonlinear least squares analysis of fluorescence recovery data yielded the diffusion coefficient and fraction of fluorescently labeled molecules that were free to diffuse.

Polarized fluorescence depletion was used to measure the rotational mobility of eosin-labeled band 3, as described.³⁰ Briefly, recovery of fluorescence excited by parallel and perpendicular probe beams was measured after a ground state depletion pulse. Band-3 rotational relaxation times were obtained from decay of fluorescence anisotropy curves, and the fraction of rotationally immobile band-3 molecules was obtained from the residual anisotropy. Data were fitted by nonlinear least squares analysis to the equation:

$$r(t) = r(\infty) + \alpha \exp(-t/\tau_1) + \beta \exp(-t/\tau_2)$$

where $r(t)$ was the anisotropy at time t , $r(\infty)$ was the residual anisotropy, and α and β were the fractions of molecules with rotational correlation times τ_1 and τ_2 , respectively.

Polymerization delay times. Blood was shipped to the National Institutes of Health (Bethesda, MD) on ice and stored at 4°C. Delay time measurements began within 24 hours of drawing the blood sample and were complete within 72 hours (there was no noticeable change in the data over this period). RBCs were washed and suspended in a solution consisting of 95 mmol/L NaCl, 20 mmol/L KCl, 23.3 mmol/L NaHCO₃, 1 mmol/L Na₂HPO₄, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 5 mmol/L glucose, and 1 mg/mL bovine serum albumin. The osmolality of this solution was previously adjusted to 290 mOsm with a solution of 1 mol/L NaCl, and 1 mol/L KCl

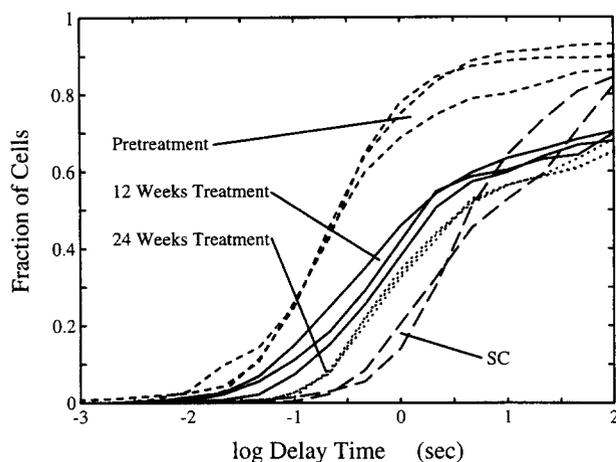


Fig 1. Fraction of sickling cells as a function of time after initiating intracellular polymerization by complete photodissociation of CO. The fraction of cells with delay times shorter than a given time is plotted versus the logarithm of the time. For each patient, three curves are shown. (---), pretreatment control; (—), 12 weeks after beginning hydroxyurea treatment; and (· · · ·), 24 weeks after beginning hydroxyurea treatment. Also shown are the results for two (untreated) patients with HbSC disease (— — —).

using an (Osmette) freezing point depression osmometer. The cell suspension was then saturated with a humidified mixture of carbon monoxide (CO) and carbon dioxide (CO₂), and the pH was adjusted to 7.35 at 37°C by varying the fraction of CO₂ (about 5%). The sample was transferred under the CO/CO₂ atmosphere to the sample cell for kinetic measurements (a Dvorak-Stottler flow cell).^{33,34} Kinetic measurements were performed until the age of the sample at 37°C was 5 hours. A previous study showed that the 2,3-diphosphoglycerate (DPG) levels were stable for more than 6 hours at 37°C in this suspending medium.³⁴

The method used for delay time measurements is based conceptually on two properties of the CO complex of HbS. The first is that a CO-saturated HbS solution does not polymerize. This insures that, with the possible exception of a very small percentage of cells, cells saturated with CO contain a liquid Hb solution with no polymerized Hb that might "nucleate" and thereby accelerate polymerization.³⁴ The second property is that a completely unliganded solution of HbS inside a single RBC can be rapidly prepared by photodissociation of the CO with a laser.^{33,35} The same laser can be used to monitor the formation of polymers from the associated increase in scattered light and, therefore, to measure the delay time before the onset of Hb polymerization. The details of the method used here for measuring delay times are very similar to those previously described.³⁴ The time course of the scattered light after photodissociation of the CO complex with a continuous argon ion laser was measured on 200 to 450 cells for each blood sample at 37°C. The light-scattering signal was collected on each RBC for 100 seconds or until polymerization was apparent, whichever was shorter. The delay time was taken as the time of onset of polymerization, as measured by the sudden increase in light scattering.

RESULTS

Hydroxyurea increases sickle Hb polymerization delay time. Figure 1 shows the fraction of cells with delay times of intracellular polymerization shorter than a given time for the three patients. We shall use the term "sickling" to indi-

cate that intracellular polymerization is occurring, with or without a change in cell morphology. For each patient, control data sets were obtained before the administration of hydroxyurea, a second data set was obtained 12 weeks after the onset of treatment, and a third set was obtained after 24 weeks of treatment. Because the distribution of delay times spans more than 5 orders of magnitude, the data are shown as the logarithm of the delay time. The similarity of these median delay times is most probably a fortuitous result.³³

Figure 2A is a plot of the median delay time as a function of time after the beginning of hydroxyurea treatment. The median delay time is the delay time at which 50% of all cells, including the "nonsickling" cells, undergo intracellular polymerization. Nonsickling cells are defined as cells showing no polymerization within the 100-second period of laser illumination. For these cells, we assume that the delay time was longer than 100 seconds or that the total concentration of Hb in the cell did not exceed the solubility, meaning that polymerization could never occur.

Before treatment, the median delay time is 0.3 second for all three patients. For each patient, the median delay time increases by at least fourfold after 12 weeks of treatment and by about another twofold during the second 12 weeks of treatment (Fig 2A). The change in median delay time arises from two effects, an increase in the fraction of nonsickling cells and a decrease in the fraction of the most rapidly sickling cells. The increase in the median delay time during the first 12 weeks results both from a decrease in the most rapidly sickling cells and from an increase in the fraction of nonsickling cells. In contrast, there is a suggestion that the increase in the median delay time during the second 12 weeks of treatment results more from a decrease in the most rapidly sickling cells.

Figure 3 compares the data on the fraction of dense cells and the fraction of rapidly sickling cells, defined here as cells with delay times shorter than 0.1 second. In all three patients, there is a monotonic decrease in the fraction of rapidly sickling cells after the start of hydroxyurea treatment (Fig 3A). In contrast, the fraction of dense cells decreases monotonically in one patient, decreases and then increases in the second patient, and barely changes in the third (Fig 3B).

Delay time distributions were also measured on RBCs from two patients with HbSC disease. Figure 1 compares these distributions with those of the three patients on hydroxyurea therapy. After 24 weeks of hydroxyurea therapy, the median delay times of the cells from the patients with homozygous sickle cell disease approached the values of the patients with HbSC disease. However, the HbSC cells have sharper distributions, with few nonsickling cells and fewer rapidly sickling cells.

Hydroxyurea reduces erythrocyte adhesiveness. Before treatment with hydroxyurea, the mean number of RBCs adherent to endothelial cells per square millimeter of endothelial surface is 50 for the three patients (Fig 4). Intrinsic patient-to-patient variability occurs in adhesion data of this nature. This level of adhesion is higher than that typically observed in our studies of other sickle cell patients (as high as 40 to 60 or as low as 1 to 2 RBC/mm²), but not strikingly so.

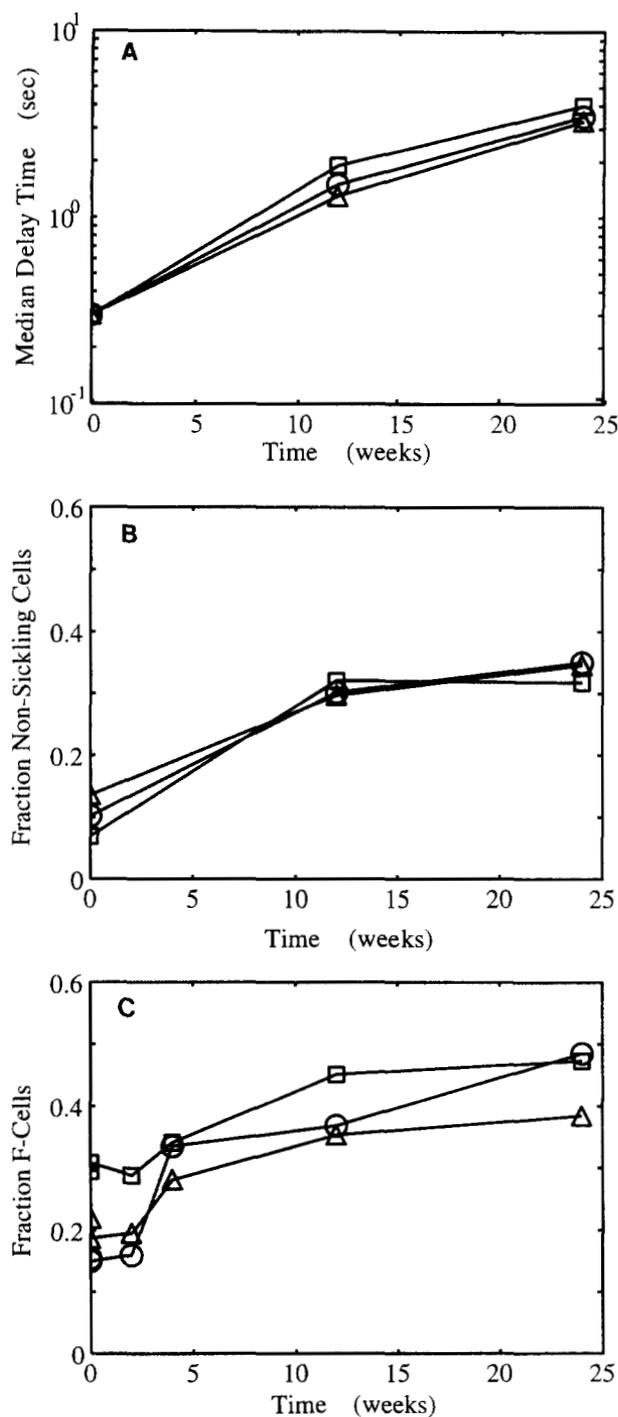


Fig 2. Correlation of delay times and F cells. The data for the three patients are labeled as follows: (○) patient A; (□), patient B; (△), patient C. (A) Median delay time as a function of treatment time. The median delay time is defined as the time at which 50% of all cells begin to undergo intracellular polymerization. (B) Fraction of nonsickling cells, defined as the fraction of cells that show no polymerization in less than 100 seconds, as a function of treatment time. (C) Fraction of F cells as a function of treatment time.

Sickle RBC adhesion to endothelial cells is significantly higher than that observed for normal RBCs (0 to 4 RBC/mm²). Adherence decreased to normal RBC values in all three patients at the first data point, 2 weeks after the start of hydroxyurea treatment. The adhesion measurements continued in the normal range for the remaining 22 weeks of the study (Fig 4). Data are the mean \pm SEM of the number of sickle RBCs adherent per square millimeter of endothelium for 48 fields counted in two experiments, except for pretreatment data for patient C (24 fields counted in one experiment).

Hydroxyurea lowers the ¹H-NMR transverse relaxation rates of sickle RBCs. Pretreatment RBC relaxation rates significantly exceed control values. One month into treatment (the first assessment of this parameter), the relaxation rates of all patient samples decreased toward control values. Relaxation rates decreased throughout the study but never reached the normal range (Fig 5). The time course of the change in relaxation rates paralleled the increase in the percentage of HbF cells and HbF reticulocytes. Relaxation rates correlated strongly with the percentage of cells with densities

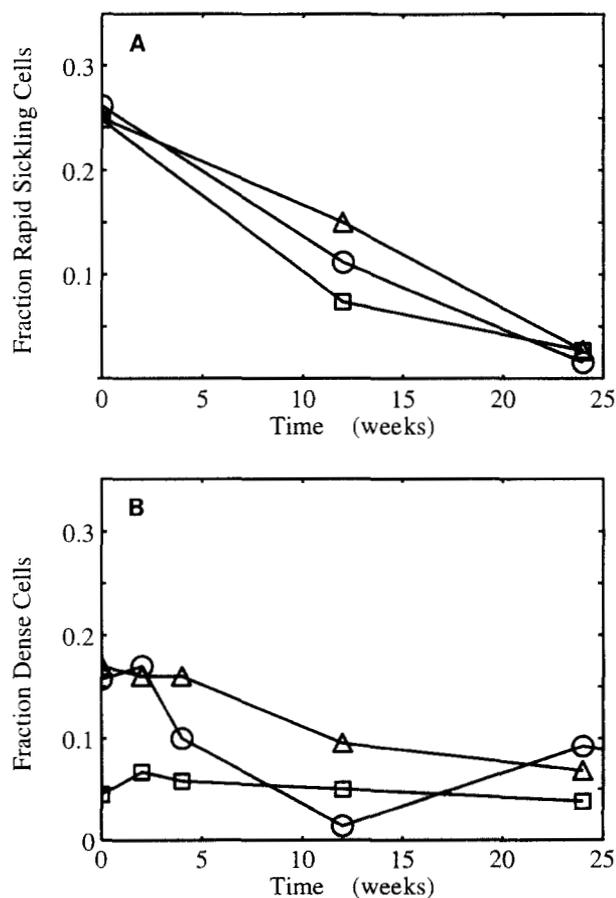


Fig 3. Fraction of rapidly sickling cells and dense cells as a function of treatment time. (A) Fraction of rapidly sickling cells defined as cells with delay times shorter than 100 milliseconds; (B) fraction of dense cells. The data for the three patients are labeled as follows: (○), patient A; (□) patient B; (△), patient C.

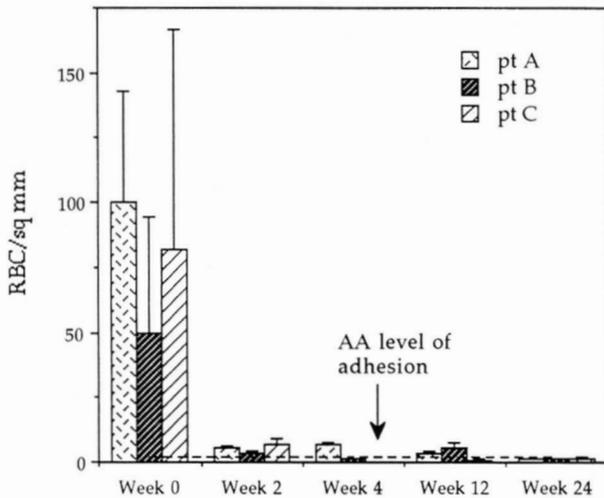


Fig 4. Effect of hydroxyurea on endothelial cell adhesion of RBCs from patients with sickle cell disease. The mean number of adherent RBCs per square millimeter of endothelium in 24 microscope fields is plotted \pm SEM. Two assays were performed before treatment, with posttreatment measurements at the times indicated. The dashed horizontal line indicates the upper value of the mean adherence of normal RBCs during the study.

higher than 1.112 (correlation coefficient was .903, $r^2 = .815$; see Fig 5B).

Hydroxyurea alters K-Cl cotransport and K^+ content of sickle RBCs. Neither ion content nor transport changed significantly during the first 4 weeks of therapy. The RBC K^+ content increased in two patients at 12 weeks and in all three patients at 24 weeks (Fig 6). After 12 weeks of hydroxyurea therapy, the K-Cl cotransport rate in all three patients decreased measurably, compared with baseline values (Fig 6). K^+ transport through the K-Cl cotransporter and the percentage of reticulocytes correlated positively ($r = .49$, $n = 17$, $t = 2.17$, $P < .05$). Hydroxyurea therapy did not alter the magnitude of the Ca^{2+} -activated ^{86}Rb influx (ie, Gardos channel; data not shown). RBC density measurements using the phthalate density profile technique showed no significant change in D_{50} (data not shown) and a lower percentage of dense cells (Table 1). Values for the R_{60} decreased in two of the three patients.

Lateral and rotational mobility of membrane-spanning proteins are unaffected by hydroxyurea. Figure 7A shows the effect of hydroxyurea treatment on the fractional mobility of band 3 in SS RBCs from three different density fractions. At week 0, as previously reported,³⁰ high-density cells showed nearly complete lateral immobilization of band 3, whereas low-density cells had band-3 mobility similar to that of normal RBCs. The band-3 fractional mobility in low-density, medium-density, and high-density cells was unaffected by hydroxyurea treatment. Glycophorin fractional mobilities, as with band-3 fractional mobilities, were unaltered by hydroxyurea treatment. Lateral diffusion coefficients of band 3 and glycophorins were 1 to 2×10^{-11} cm^2/s and 2 to 3×10^{-11} cm^2/s and were unaffected by hydroxyurea treatment.

Figure 7B and C shows the effect of hydroxyurea treat-

ment on the fractions of band 3 in rapidly rotating, slowly rotating, and rotationally immobile populations of molecules in low-density and high-density cells. At week 0, as previously reported³⁰ high-density cells showed significant rotational immobilization of band 3, whereas low-density cells had rapidly rotating, slowly rotating, and rotationally immobile populations similar to those of normal cells. The relative proportions of band-3 molecules in rapidly rotating, slowly rotating, and rotationally immobile populations did not change with hydroxyurea treatment. Rotational correlation times for rapidly rotating and slowly rotating populations were 50 to 250 microseconds and 1 to 3 milliseconds, respectively, and were unaffected by hydroxyurea treatment.

DISCUSSION

Hydroxyurea, the only drug proven to decrease the frequency of pain crises in patients with sickle cell disease,

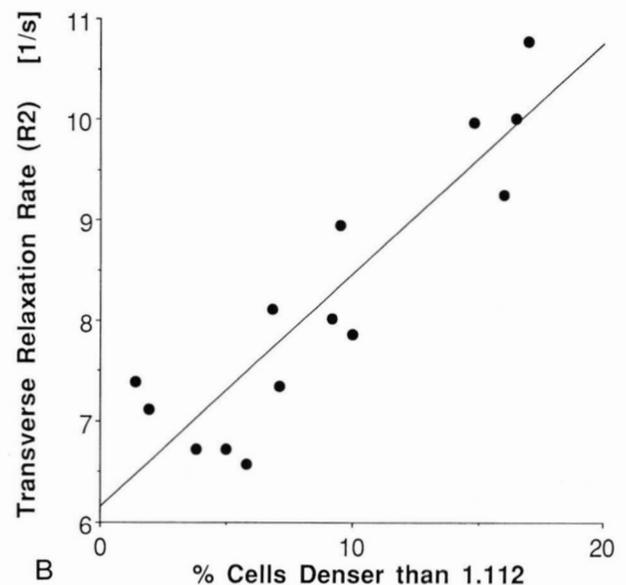
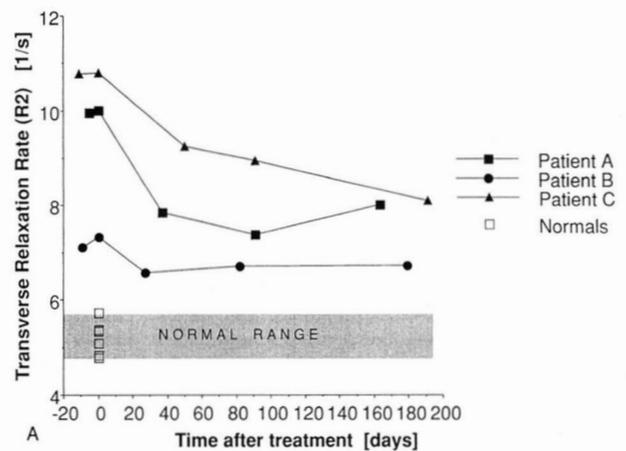


Fig 5. Effect of hydroxyurea on 1H -NMR relaxation rates in three patients. (A) Relaxation rates (R^2) are plotted as a function of treatment duration. The relaxation rates of the patient samples exceed the control values at all assay points (correlation coefficient, .9); the bars represent the SEM. (B) Relaxation rates plotted against the percentage of cells with densities greater than 1.112.

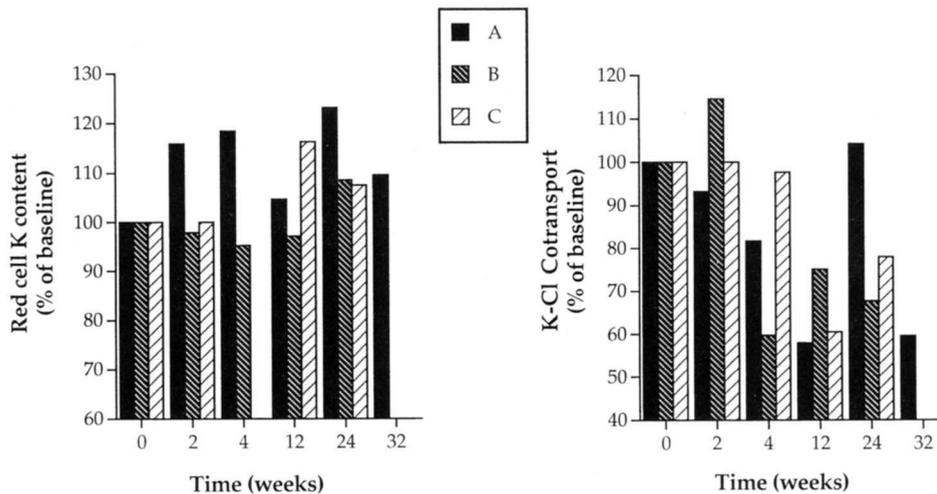


Fig 6. Effect of hydroxyurea therapy on RBC K content and K-Cl cotransport in three patients (A, B, and C) with sickle cell disease. RBC Na^+ and K^+ content was determined after washing the cells 5 times in a solution containing 140 mmol/L choline chloride, 1 mmol/L MgCl_2 , and 10 mmol/L Tris-MOPS (pH, 7.40) at 4°C. K-Cl cotransport was determined as Cl-dependent K^+ efflux in hypotonic medium in the presence of ouabain (0.1 mmol/L) and bumetanide (10 $\mu\text{mol/L}$). Nitrate was used as chloride substitute. D_{50} , R_{60} , and percent of cells denser than 1.112 were measured with the phthalate density profiles.

increases HbF levels in many patients by a still unknown mechanism. The increase in HbF manifests primarily as more numerous F cells, which nearly double after 12 to 16 weeks of hydroxyurea therapy.²¹ The earliest effect in our study was the striking decrease in RBC adhesion to endothelial cells after 2 weeks of hydroxyurea therapy (Fig 4). This change preceded the increase in HbF production in these

patients (Table 2). Therefore, hydroxyurea alters some RBC characteristics independently of its capacity to induce HbF synthesis.

Adhesion of sickle RBCs to endothelial cells in a laminar flow system depends on a number of variables. These include the expression of adhesive molecules on the RBC membrane,³⁶ soluble adhesive glycoproteins,³⁷ and adhesive glycoproteins on the membrane of the endothelial cells. The latter two variables were fixed in our system, because we used a standard resuspension system for the RBCs and standard culture conditions for the endothelial cells. Therefore, the dramatic reduction in adhesion of sickle RBCs 2 weeks into a course of treatment with hydroxyurea was caused by a reduction in the "adhesive qualities" of the RBC membrane. The suggestion of a decrease in the reticulocyte number after 2 weeks of hydroxyurea therapy (Table 1) was clearly evident at 4 weeks. Reticulocytes are particularly active in adhesion assays.³⁸ Fewer reticulocytes and, possibly, changes in the intrinsic adhesiveness of these cells could account for these data.

Interestingly, the adhesion of untreated HbSC RBCs to endothelium in this experimental set-up was quite low. From the standpoint of "adhesiveness," then, hydroxyurea treatment causes the RBCs of patients with sickle cell disease to behave similarly to those people with HbSC disease. This trend toward the RBC characteristics observed with HbSC disease parallels changes in delay time parameters (see below). The reduction in sickle RBC adhesion to endothelial cells observed with hydroxyurea therapy would facilitate movement of RBCs through the capillary bed before sickling occurs. The effect of hydroxyurea on the RBC adhesion profile would be even more striking should this drug also lower endothelial adhesiveness in vivo as occurs with bovine endothelial cells in vitro.³⁹

Other parameters that reflect membrane injury in sickle RBCs are the lateral and rotational mobility of membrane-spanning proteins. Hydroxyurea does not correct the abnormalities³⁰ associated with sickle cell disease. Although hydroxyurea therapy significantly shifts the density distribution of sickle RBCs, the remaining high-density cells manifest a

Table 1. Effect of Hydroxyurea on Erythrocytes and Reticulocytes in Three Patients With Sickle Cell Disease

	Baseline	Treatment time (wk)			
		2	4	12	24
MCV (fL)					
A	90	94.1	103.1	112.6	105.6
B	96	105.8	101.9	112.7	103.6
C	98	103.6	110.5	109.9	106.7
MCV _r (fL)					
A	105.5	115.5	115.9	122.1	133.6
B	107.5	120.8	118.2	122.8	112.1
C	120.3	117.7	132.0	123.9	116.8
HDW (g/dL)					
A	4.15	3.87	3.57	3.73	3.68
B	3.15	3.62	2.93	2.81	3.15
C	4.50	4.00	4.16	3.53	3.78
R_{60}					
A	0.014	0.012	0.009	0.008	0.008
B	0.008	0.012	0.009	0.008	0.008
C	0.019	0.012	0.012	0.010	0.010
% Dense cells					
A	15.7	16.7	10.0	9.2	6.5
B	4.5	6.7	5.8	5.0	2.0
C	17.0	16.0	16.0	9.5	6.8
Reticulocytes ($\times 10^9/\text{L}$)					
A	237	184	117	104	57
B	272	289	113	106	198
C	239	192	98	85	99

Values shown are for patients A, B, and C.

Abbreviations: MCV, mean corpuscular volume; MCV_r, mean corpuscular volume (reticulocyte); HDW, Hb distribution width.

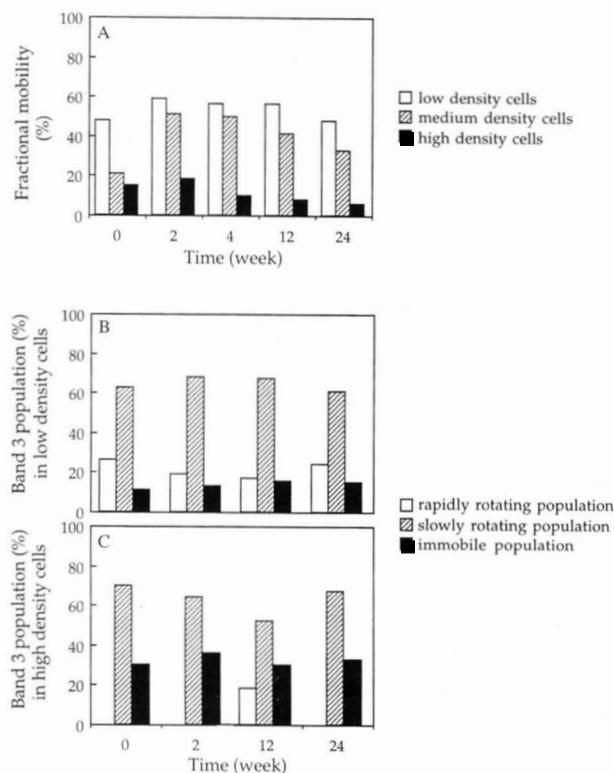


Fig 7. Effect of hydroxyurea treatment on lateral and rotational mobility of band 3 in density-fractionated sickle RBCs. Lateral mobility was determined by the fluorescence photobleaching recovery technique, and rotational mobility was determined by the polarized fluorescence depletion technique. Laterally (A) and rotationally (B and C) mobile fractions of band 3 were measured in density-fractionated sickle RBCs at various times after initiation of hydroxyurea treatment. (A) Values represent mean band-3 fractional mobility in 10 independent experiments on cells from a single individual. Standard deviations were 3% to 16% of mean values. (B and C) Values represent band-3 rotational populations in low- (B) and high- (C) density fractions of cells from a single individual. Rotational mobility data were averaged over about 500 cells per measurement. Band-3 populations were obtained by fitting anisotropy decay data to the mathematical function described in the text. Data from the other two individuals in the study differed insignificantly from those shown in this figure.

degree of band 3 and glycophorin immobilization comparable with that observed in high-density cells from untreated patients. Transmembrane protein immobilization in high-density cells is likely caused by acquired, irreversible membrane abnormalities including protein clustering and oxidative cross-linking.³⁰ Hydroxyurea appears not to prevent such membrane damage but, rather, to limit the fraction of cells that manifest such injury.

Repetitive cycles of sickling damages RBC membranes.^{40,41} One consequence is enhanced K-Cl cotransport and lower RBC K⁺ content. Consistent with our previous observations, hydroxyurea treatment countered these changes, increasing cellular K⁺ content and lowering K-Cl cotransport (Fig 6). These transport effects of hydroxyurea parallel induction of HbF synthesis (Table 2), suggesting that they reflect less repetitive sickling and less membrane

damage. The Gardos channel is unaffected by hydroxyurea. Agents that specifically alter this membrane pump could further increase cellular K⁺ content and reduce the number of dense cells. When the transport data are analyzed with an eye to the lateral and rotational mobility results, a picture emerges in which hydroxyurea limits the fraction of cells that sustain membrane injury, thereby reducing both the fraction of cells with low K⁺ content and with impaired lateral and rotational mobility of membrane-spanning proteins. Our data do not indicate directly whether the same cells are in these functionally defined fractions. However, overlap is likely because dense cells are probably the major component of these "damaged" cells.

Hydroxyurea induces changes in the Hb polymerization delay time synchronously with enhanced HbF production. F cells contain about 20% HbF, 80% HbS, and more total Hb than do normal cells. However, the total intracellular Hb concentration is approximately normal because of their greater volume.^{42,43} The dilution of HbS by HbF dramatically increases the delay time. Polymerization studies of HbS solutions show an approximate 1,000-fold increase in the delay time.⁴⁴ Therefore, the vast majority of F cells should have delay times that exceed 100 seconds, which we have defined as nonsickling cells. This explains the close correlation between the fraction of nonsickling cells and the fraction of F cells (Figs 2B and C).

The probability is low that the nonsickling cell population contains a significant fraction of cells with no HbF (S cells). Using the data on HbS solutions,^{33,35} cells that show delay times longer than 100 seconds and contain only HbS would have intracellular HbS concentrations of less than 23 g/dL. This is lower than the lowest values obtained by density measurements of the intracellular Hb concentration distribution. Therefore, we conclude that the nonsickling RBC population consists almost entirely of F cells. This conclusion predicts that the fraction of nonsickling cells represents a lower limit on the fraction of F cells, which is consistent with the data, as shown in Fig 2B and C. Thus, the observation that the fraction of F cells exceeds the fraction of nonsickling cells by about 25% (Fig 3) presumably reflects dehydration (higher Hb concentration) of some F cells that are entrapped and sickle in a "log-jam" initiated by S cells.

These delay times are measured with completely deoxygenated (laser-deligated) solutions. At partial saturation with oxygen, as occurs in vivo, all delay times would be much longer.⁴⁵ Even cells containing no HbF would have delay times much longer than the transit time through the microcirculation.³⁴ In some F cells, the solubility may exceed the total Hb concentration, making polymerization thermodynamically impossible.^{8,10,46} The picture that emerges has cells with HbF passing through the microcirculation with a much lower probability of sickling than cells without HbF ("S cells"; Fig 8).

The fraction of rapidly sickling cells decreases monotonically during hydroxyurea treatment, whereas the fraction of dense cells varies much less systematically. The close correlation between the data on NMR transverse relaxation time and the fractional representation of dense cells means that ¹H-NMR relaxation rates are surrogate markers of dense

Table 2. Effect of Hydroxyurea on F-Cells and F-Reticulocytes in Three Patients With Sickle Cell Disease

Patient	Pretreatment	Pretreatment	2 Wk	4 Wk	12 Wk	24 Wk
A						
% F-retics	4.7	4.1	5.3	12.0	11.3	23.3
% F-cells	15.5	15.0	16.0	33.5	36.8	48.5
B						
% F-retics	14.9	15.6	—	24.0	22.7	24.1
% F-cells	29.5	30.8	28.8	34.1	45.1	47.3
C						
% F-retics	10.0	9.2	11.1	16.7	26.0	28.0
% F-cells	21.9	18.7	19.5	28.1	35.3	38.4

cells (Fig 5B.) Therefore, NMR transverse relaxation time has promise as a method for monitoring changes in the dense cell population. This technique provides faster and easier measurements than do density gradient profiles, and the set-up is relatively simple.

Why is the correlation between dense cells and rapidly sickling cells so poor (Fig 3)? The original motivation for monitoring the dense cell population came with the realization of the enormous sensitivity of the kinetics of polymerization to intracellular Hb concentration (in contrast to the weak dependence on concentration of the equilibrium fraction polymerized).^{47,48} Dense cells have the highest probab-

ity of sickling in vivo, because the kinetics of polymerization is most rapid in these cells. Measuring dense cells is much easier than measuring the fraction of rapidly sickling cells, possibly making it a useful surrogate marker. However, the present data suggest that density does not faithfully reflect speed of polymerization in patients with numerous F cells. The most apparent explanation of the poor correlation is that the dense cell fraction contains F cells with much longer delay times than S cells of the same total concentration (density). This raises the more general question of whether dense cells are a reliable indicator of rapidly sickling cells. Further investigation of this question is clearly warranted.

Assuming that the distribution of delay times faithfully reflects clinical severity, the similarity of the median delay times between patients with homozygous sickle cell disease treated with hydroxyurea for 24 weeks and patients with HbSC disease (Fig 1) indicates similar clinical severity. Because HbSC disease is known to be generally milder than homozygous sickle cell disease, this result would argue for a significant clinical benefit of hydroxyurea. Using delay time criteria alone, a "cure" would be expected if the minimum delay time at complete deoxygenation were 10 seconds, corresponding to that observed in sickle cell trait.^{10,46} However, the minimum delay time both in patients with HbSC disease and in hydroxyurea-treated patients with homozygous sickle cell disease is less than 0.1 second.

The argument can be taken one step further by examining the distribution of delay times. The distribution is narrower in patients with HbSC disease, where both fewer nonsickling cells and fewer cells with delay times less than 1 second are observed. The likelihood is low that without entrapment in a "log-jam," cells with delay times greater than about 1 second at complete deoxygenation ever undergo polymerization at partial saturation in the microcirculation (with the possible exception of the hypertonic renal medulla). Because the more rapidly sickling cells are most likely to trigger or participate in occlusions in the microcirculation, we tentatively conclude (given the small number of patients) that hydroxyurea treatment would ameliorate the clinical picture of sickle cell disease toward that of HbSC disease. The lower adhesion between sickle RBCs and endothelial cells toward the pattern observed with HbSC cells also argues for amelioration of sickle cell disease with hydroxyurea therapy. The degree of clinical improvement observed in the Multicenter Study of Hydroxy-

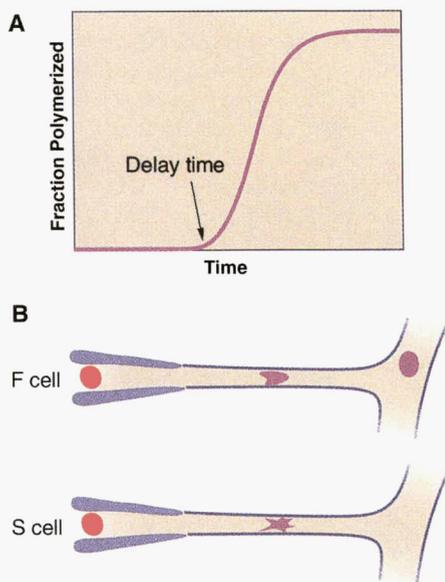


Fig 8. Delay time of HbS polymerization and transit of RBCs through the microcirculation. (A) Kinetic progress curve for the polymerization of HbS. **(B)** In the upper panel, an RBC containing HbF (F cell) passes through a capillary and escapes without intracellular polymerization because the delay time is longer than the transit time; in the lower panel, the delay time for a cell containing no HbF (S cell) is shorter than the capillary transit time, and polymerization and cellular deformation ("sickling") occur while the cell is in the capillary, with the possibility of occluding the vessel. (Reprinted with permission from Eaton W, Hofrichter J: The biophysics of sickle cell hydroxyurea therapy. *Science* 268:1142, 1995.⁹ Copyright 1995 American Association for the Advancement of Science.)

urea in Sickle Cell Anemia (MSH) trial is consistent with these phenotypic changes. The lack of measurable clinical improvement in some patients in the MSH study²⁰ is also consistent with this analysis.

In summary, with the exception of the unexpected effect on erythrocyte adherence, the changes in the parameters assayed in this study are consistent with an increase in F-cell representation as the principally important effect of hydroxyurea. Changes in the fraction of slowly or nonsickling cells and the mean K⁺ content derive from this effect. Both the change in distribution of delay times and the lower RBC "adhesiveness" suggest that hydroxyurea treatment will reduce the severity toward that of HbSC disease, but this conclusion will obviously remain a tentative one until many more patients have been studied.

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