Some Aspects of the Pathophysiology of Homozygous Hb CC Erythrocytes

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ABSTRACT We have studied erythrocytes from homozygous CC patients in vitro and in perfused rat mesoappendix vasculature to answer some long-standing questions. By examination of wet whole blood preparations, and by comparing the cell distribution on isopycnic continuous density gradients of whole blood samples from a splenectomized CC patient with those from three intact CC patients, we have demonstrated the presence of a distinct crystal-containing band of cells that is present in the former, but totally absent from the latter. We conclude that Hb CC cells containing crystals circulate in Hb CC individuals, but in intact patients they are effectively removed by the spleen. By use of ^{31}P nuclear magnetic resonance and viscosity measurements on cells, we have demonstrated that intracellular aggregation of hemoglobin C occurs on deoxygenation even when no crystal formation is detectable by morphological methods. These two observations are in apparent contradiction with the absence of clinical microcirculatory impairment found in both intact and splenectomized CC patients. The contradiction was resolved by rheological studies on isolated rat mesoappendix preparations and erythrocyte diameter measurements that lead to the conclusion that the hemorheological properties of CC cells in the microcirculation are nearly normal because their increased viscosity is offset by their smaller diameter and size.

INTRODUCTION

Hemoglobin C was discovered by Itano and Neel (1) in an individual heterozygous for Hb, S, and the first cases of homozygous Hb C were described a few years later by Spaet et al. (2) and Levin et al. (3).

Understanding of the pathophysiology of Hb CC disease was advanced by the classical observations of Charache et al. (4) and by the work of Murphy (5) and Lessin et al. (6). Nevertheless, several issues remain unresolved.

One pending question is the presence or absence of Hb C crystals in circulating cells. Since the earliest descriptions of cases of Hb CC disease, intracellular crystals, have been detected in wet preparations of saline suspended cells, in dry and stained smears and, very readily, in 3% NaCl suspensions of erythrocytes (6–8). Of course, none of these observations answers the question posed, as varying degrees of in vitro erythrocytes shrinkage either were undoubtedly present or cannot be conclusively excluded. We will report here our studies on native whole blood of a splenectomized Hb CC patient in order to resolve this question.

The next outstanding question pertains to the detection of intracellular aggregation of Hb C (before crystals can be observed). No knowledge exists of its occurrence in cells, or of the time scale of this process, nor of its ligand dependency. The lack of information is due to the absence of appropriate methodology. We have previously used ^{31}P nuclear magnetic resonance, (NMR)¹ line broadening (T_2 effects) to study hemoglobin S polymerization (9) and will report here the application of the same technique to the observation of Hb C aggregation.

Finally, there is the apparent contradiction between the benign clinical course of the disease and the abnormal viscous properties exhibited by Hb CC erythrocytes. These erythrocytes have been found to be less

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¹Abbreviations used in this paper: DPG, diphosphoglycerate; MCHC, mean corpuscular hemoglobin concentration; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PRU, peripheral resistance units; RBC, erythrocytes.

filterable (4) and to exhibit equally elevated viscosity in both the oxy- and deoxygenated states as compared to Hb AA cells (5, 10, 11). In a previous publication (11), we described the use of the perfused rat mesoappendix to study flow properties of abnormal erythrocytes. In this publication, we present data on the effect of splenectomy and deoxygenation on hemodynamics and rheology of Hb CC cells.

METHODS

Blood samples, characterization, and morphological studies. Samples of heparinized blood were obtained by venipuncture or finger stick from four Hb CC homozygote patients after informed consent was received. One of these patients had undergone splenectomy 10 yr ago.

The percentage of oxy-, carbon monoxy-, and deoxy-hemoglobin present in the samples was measured using an Instrumentation Laboratory CO-oximeter model 282. (Instrumentation Laboratory, Inc., Lexington, Mass.) Measurements were made in duplicate or triplicate and reproducibility was $\pm 2\%$ of the stated values. For the hemodynamic studies, PO_2 of the samples was determined with a blood gas monitor and PHM-27 (Radiometer Laboratories, Copenhagen, Denmark).

Direct and exhaustive examination of the erythrocytes of these patients was performed in wet preparations of undiluted whole blood using a Nomarski differential interference contrast microscope (Carl Zeiss, Inc., New York).

Preparation of samples for scanning electron microscopy involved fixation in 10% formaline in phosphate-buffered saline, layering of cells on 0.45- μm filters (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and dehydration through series of increasing ethanol concentrations followed by Freon 113. Freon 13 was used as transitional fluid for critical point drying (Bomar SPC-900/EX, The Bomar Co., Tacoma, Wash.) and gold sputtering for coating (EMS-41 Minicoater, Film-Vac Inc., Englewood, N. J.). Finally, samples were examined on a MiniSEM (International Scientific Instruments, Inc., Santa Clara, Calif.).

The samples were deoxygenated by alternate application of a moderate vacuum and water-saturated nitrogen and were rotated to ensure a large surface area for gas exchange. Partially deoxygenated samples were obtained directly rather than by mixing oxy- and deoxy- solutions or by reoxygenating deoxygenated solutions. Transfers to NMR tubes or other containers were made under a nitrogen atmosphere in a glove bag.

Isopycnic gradients for erythrocyte separation. Percoll-Renograffin gradients were run as described by Vettore et al. (12), and were prepared using a ratio of Percoll/Renograffin-76/water/0.9% NaCl equal to 3.5:1.7:4.3:0.7. The resulting osmolality was 320 mosmol and the pH was 7.5. For anemic patients the hematocrit was adjusted to 40–45 before the cells and plasma were added to the gradient mixture. For analytic studies, 0.1 to 0.2 ml of whole heparinized blood was added to 5.9 or 5.8 ml of gradient mix. The tubes were spun for 20 min at 17,000 rpm (35,000 g) in a Sorvall SS-34 rotor (DuPont Instruments, Sorvall Operations; Newtown, Conn.).

NMR measurements. A Jeol PFT-100 Fourier transform NMR (Jeol USA Inc., Peabody, Mass.) operating at 40.48 MHz was used for these measurements. T_1 was measured using a $180^\circ\text{-}\tau\text{-}90^\circ\text{-AQT}$ pulse sequence. T_2 was measured using $90^\circ\text{-}\tau\text{-}180^\circ\text{-}\tau\text{-AQT}$. The nuclear Overhauser effect was measured using gated decoupling and delay times as described by Opella et al. (13). All other spectra were collected with continuous broad band proton decoupling. Samples were contained in 10-

mm tubes with a Wilmad coaxial inner tube (Wilmad Glass Co., Inc., Buena, N. J.) that contained D_2O for a lock and 5 mM D_3PO_4 in 0.1 M D_2SO_4 as a reference. It is necessary to isolate the D_2O for the lock from the hemoglobin solutions, since aggregation might result in a dramatic increase in the D_2O linewidth. The concentric tubes also provide an oxygen-tight seal that is effective for several days. All measurements were made at 25°C .

Viscometry. Viscosity measurements were made at 37°C on oxygenated and deoxygenated blood at a hematocrit of 30%. A Wells-Brookfield micro cone-plate viscometer (model LVT, Brookfield Engineering Laboratories, Inc., Stoughton, Mass.) was used. Calibration of cone and cup was done frequently using a silicone oil standard (viscosity-5 centipoise). Each time 1.10 ml of sample was pipetted into the center of the cup and viscosity measurements were taken in duplicate at shear rates ranging from 5.75 to 230 s^{-1} . During the study of deoxygenated blood samples, the cone-plate chamber was gassed with N_2 via the gas inlet. Samples taken before and after viscometric measurements showed $<2\%$ change in oxygen saturation.

Hemodynamic studies. These studies were performed in an isolated, acutely-denervated, artificially perfused rat mesoappendix vasculature (14). In anesthetized (pentobarbital sodium 30 mg/kg) rats of the Wistar strain, 120–150 g, the right ileo-colic artery and vein were cannulated. After the cecum and terminal ileum (each 3 cm long) were ligated and hemostasis of all other vascular connections to the organ was achieved, the tissue was isolated, and perfused with mammalian Ringer's solution containing 1.5% bovine albumin at pH 7.4 and $37 \pm 0.5^\circ\text{C}$, and gassed with 95% – 5% CO_2 or N_2 . The isolated tissue, covered by a lucite capsule, was also continuously suffused with Ringer's solution (containing 1.0% gelatin) of the same pH and temperature. Arterial perfusion (Ppa) and venous outflow pressures (Pv) were kept constant at 80 and 3.9 mm Hg, respectively. The venous outflow (Fv) rate was monitored using an electric dropcounter and expressed in milliliters per minute. A lapse of 10–12 min was allowed for tissue equilibration and stabilization of Fv. Only preparations exhibiting mesoappendix microvasculature free of blood cells and with a steady Fv of 2.8 ± 0.5 ml/min (mean and SD) were used. After control measurements of Ppa and Fv, oxygenated or deoxygenated blood samples (0.2 ml, vol) of 30% hematocrit were gently delivered via an injection port, 15 cm distal to site of arterial cannulation, and the changes in Ppa and Fv were recorded on the strip chart of a Grass polygraph (Grass Instrument Co., Quincy, Mass.) Each mesoappendix and tissue vasculature preparation was sufficient for the evaluation of four to five samples. However, routinely, administration of an Hb CC blood sample was preceded or followed by the same volume of Hb AA of matched hematocrit at intervals of 8–10 min. When deoxy conditions were used, the O_2 saturation of erythrocytes before and after passage through the microvasculature was determined and showed a change of $<10\%$. The tissue preparations were perfused for 30–45 min prior to these experiments with Ringer's solution that had been deoxygenated by bubbling nitrogen through it. At the end of each experiment the entire tissue preparation (free of cannulas and luminal content) was weighed. Peripheral resistance units (PRU) were calculated as previously described (15) and expressed as $\text{PRU} = \Delta P/\dot{Q}$ = millimeters of Hg per milliliter per minute per gram; where ΔP (millimeters Hg) is the arteriovenous pressure difference and $\dot{Q}$ (milliliters per minute per gram) is the rate of venous outflow per gram of the tissue.

Erythrocyte diameter measurements. The diameters of Hb AA and Hb CC erythrocytes were measured in whole blood and from top and bottom density gradient fractions. Erythrocyte suspensions were made in isotonic phosphate-

FIGURE 1 Nomarski optics field of a wet and unperturbed whole blood sample from a splenectomized CC patient. Notice the presence of at least three crystals in the field. This field is representative of the whole field.

buffered saline at pH 7.4. A Nomarski differential interference contrast microscope coupled with an image splitter and polygraph system (16) was used for observation. Measurements were made at an optical magnification of 1,000. In each case ~100 cell diameters were measured. Statistical analysis was done using a Tektronix 31 computer (Tektronix, Inc., Beaverton, Ore.).

RESULTS

Presence of crystals in circulating cells. Wet, unstained, and undiluted preparations of fresh whole blood from splenectomized and intact Hb CC patients were examined under Nomarski optics. Fig. 1 shows the presence of many cells containing crystals in blood obtained from the splenectomized patient. No such crystals were observed in the nonsplenectomized patients even after exhaustive examination of the slides.

When whole blood from three intact homozygous Hb CC patients and one splenectomized CC patient was separated on Percoll-Renograffin continuous density gradients (Fig. 2) two major findings were obtained: (a) The splenectomized patient had an additional dense band that was consistently absent from the gradient pattern of the other three CC patients. When observed under Nomarski optics, this band turned out to contain mostly cells with intracellular crystals. Scanning electron microscopy of this fraction is shown

FIGURE 2 Percoll-Renograffin continuous density gradients. From left to right: an intact CC patient, a normal AA control, and a splenectomized CC patient. Reticulocytes were found at the level marked "CC retics" in both the intact and splenectomized CC patient. Note the absence of the very dense band containing CC crystals in the intact CC patient. The arrows are position markers and do not necessarily imply the presence of the species indicated in any one tube.

in Fig. 3. (b) Erythrocytes from Hb CC patients were denser than normal cells (in accordance with their classical increase in mean corpuscular hemoglobin concentration [MCHC]). In addition, all Hb CC patients (splenectomized and intact) exhibited reticulocytes that were denser than normal reticulocytes (Fig. 2). Reticulocytes were identified by methylene blue staining and constituted ~35% of the band designated "retics" in Fig. 2. (c) Comparison of the density gradient pattern of a splenectomized AA patient to intact AA controls showed no difference within the normal (small) range of variation found for AA subjects. No evidence of very dense cells was found.

Intracellular aggregation of Hb C by P-31 NMR. The effect of deoxygenation on the P-31 NMR spectrum of erythrocytes from patients homozygous for hemoglobin C is shown in Fig. 4. At low oxygen tensions, a distinctive pattern of the two narrow lines of 2,3-diphosphoglycerate (DPG) (one from each phosphorus) are superimposed on a broader line or lines. The small narrow line to the left of the 2,3-DPG lines is due to inorganic phosphate (these spectra are reversed right to left due to use of a crystal filter). The line to the right of the 2,3-DPG lines is the result of sugar phosphates, which vary in concentration from subject to subject. Fresh erythrocytes (RBC) do not have NMR detectable levels of inorganic phosphate, so the presence of this signal indicates that some degradation of phosphorylated compounds has occurred.

We have measured the nuclear Overhauser effect (NOE) and T_1 and T_2 relaxation of both the broad and narrow components of the 2,3-DPG spectrum in partially deoxygenated CC cell samples. The NOE is an enhancement of the P-31 signal intensity that occurs

FIGURE 3 Scanning electron microscopy of a RBC preparation from a splenectomized CC patient. A and B: washed cells from whole blood. The arrow indicates a "folded" cell, which is an infrequent but characteristic finding in CC blood; C and D: washed cells from the band identified as "CC crystals" in Fig. 2. A, B, and D correspond to $\times 800$; C is $\times 1,000$.

when continuous broad band proton decoupling is used to simplify the spectra. We find that both components have a T_1 of ~ 2 s. This is comparable to the 2,3-DPG T_1 value found by Gupta in normal human RBC (17). A 10% enhancement was detected for the narrow component when the NOE was measured. The broad component showed no appreciable enhancement. The relative amount of aggregated Hb C can be over- or underestimated if either the T_1 or the NOE of the broad and narrow components are appreciably different. When T_2 was measured, we found that the broad component had a T_2 in the range of 3 to 5 ms, which is compatible with the estimated linewidth.

Fig. 4 shows the effects of partial deoxygenation on whole blood samples obtained from a normal AA control (A); an AA control in hypertonic media where the MCHC matched that of CC patients (B); and two homozygous CC patients (C and D). Patient C has been splenectomized and A, B, and D have not. The biphasic pattern of narrow peaks superimposed on a broad resonance observed for the CC patients is not found until relatively low oxygen tensions are reached. T_2 studies indicate that relaxation must be characterized by two or more relaxation times. This implies that 2,3-DPG is found in two environments and that the broad signal comes from P-31 in an environment characterized by slow molecular motion. The area under the curves is conserved irrespective of the as-

sumptions made about linewidths or the ratio of broad and narrow components. This implies that all of the 2,3-DPG can be detected and that deoxygenation does not result in the loss of the P-31 signal. Curve-fitting experiments indicate that two relaxation times (T_2) are both necessary and sufficient to characterize the data. The width of the broad component can be estimated from T_2 and curve-fitting, we find that a linewidth of 80 Hz is consistent with the available data. The narrow component can be estimated from curve-fitting experiments to be 12 Hz.

The broadening of the 2,3-DPG phosphate peaks observed when AA cells are deoxygenated is quite modest in comparison and could be described by a single T_2 in all cases even after the MCHC was elevated to equal that characteristic of CC cells.

It is clear from Fig. 4 that the aggregated component is more abundant in RBC from the splenectomized patient, at each deoxy hemoglobin concentration.

Viscosity of Hb CC cells and its ligand dependency. Fig. 5 shows viscosity curves for plasma suspensions of Hb AA RBC and Hb CC cells from intact and splenectomized patients at a hematocrit of 30% in both oxy- and deoxygenated states. There was a clear shear rate dependence resulting in increased blood viscosity as shear rates were reduced from 230 to 5.75 s^{-1} . Such an increase in blood viscosity at low rates of shear has been attributed to the RBC aggregation and formation

FIGURE 4 P-31 NMR spectra as a function of deoxygenation. The figure represents 14 separate NMR spectra. Each spectrum was taken at the percent deoxygenation indicated by the arrows centered between the 2,3-DPG peaks. The cells in each set of spectra came from the following sources: A, an AA control; B, an AA suspended in hypertonic medium so the MCHC equals that of CC cells; C, a splenectomized CC patient (the spectra for 0 and 62% deoxy-hemoglobin and those for 7% and 88% deoxy-hemoglobin were from the same patient but the samples were drawn 3 wk apart); D, an intact CC patient.

of rouleaux-like structure in both normal and abnormal RBC (18). It is interesting to note that in the oxygenated state the viscosity of Hb CC RBC from the splenectomized patient (splex) was significantly elevated compared to that of Hb CC (intact) blood, which, however, was much more viscous than Hb AA blood. After deoxygenation, the RBC suspension from Hb CC (splex) patient (and to a lesser degree Hb CC [intact]) showed a further increase in viscosity at all the shear rates used. In contrast there was little or no change in the viscosity curves of Hb AA blood samples following deoxygenation. A splenectomized AA patient was also studied and had a viscosity within the normal range for intact AA subjects. The viscosities of oxy- and deoxy-SS cells are included for comparison.

FIGURE 5 Relationship between viscosity (centipoise, cP) (mean values) and shear rates at a hematocrit of 30% and temperature of 37°C. (AA oxy, $\circ$; deoxy, $\bullet$; CC (intact) oxy, Δ ; deoxy, $\blacktriangle$; CC (splex) oxy, $\square$; deoxy, $\blacksquare$; SS oxy, $\diamond$; deoxy, $\blacklozenge$).

Hemodynamic studies. Table I gives the net changes in PRU percent, the mean difference of PRU [(PRU of CC cells) – (PRU of AA cells)] and the changes in pressure-flow recovery time after administration of oxygenated Hb CC RBC from intact or splenectomized individuals was only slightly higher than that for Hb AA controls. Following deoxygenation, injection of Hb CC RBC from the splenectomized patient resulted in a

TABLE I
PRU in the Isolated Mesoappendix for Hb AA, Hb CC (Intact), and Hb CC (Splenectomized) Blood at a Hematocrit of 30%

Blood		PRU	Δ PRU (CC-AA)	Δ Pressure-flow recovery time (CC-AA)
		% change		s
First prep	Oxy Hb AA	13.3		
	Oxy Hb CC (intact)	21.6	8.3	8
	Deoxy Hb AA	12.6		
	Deoxy Hb CC (intact)	25.3	12.7	8
Second prep	Oxy Hb AA	3.0		
	Oxy Hb CC (splex)	5.3	2.3	8
	Deoxy Hb AA	7.6		
	Deoxy Hb CC (splex)	28.4	20.8	28

considerably higher PRU percent increase and also an increase in pressure-flow recovery time when compared to changes resulting from the administration of Hb CC RBC from the nonsplenectomized individual.

Diameters of RBC. Table II gives the mean diameter (micrometers) of Hb AA, Hb CC (intact), and Hb CC (splex) RBC in whole blood and in the top and bottom density gradient fractions. In the whole blood samples, the mean diameter of both Hb CC (intact) and Hb CC (splex) RBC were significantly smaller than that of Hb AA cells. Comparison of the diameters of cells from the top fractions revealed a significantly larger mean diameter for Hb CC (splex) cells as compared to that of Hb CC (intact) cells, but no significant difference was noted between the diameters of Hb CC (both intact and splex) and Hb AA cells. Analysis of the diameters from the bottom fraction cell population revealed significantly smaller mean diameter for intact and splex Hb CC cells, but they were not much different from each other.

DISCUSSION

By examination of wet preparations of unperturbed fresh blood samples we have demonstrated that the splenectomized Hb CC patient has circulating crystal-containing cells. These cells form a distinct band (Fig. 2) in isopycnic density gradients that is totally lacking from the other three homozygous Hb C patients. Furthermore, exhaustive microscopic examination of wet whole blood preparations from the intact patients failed to reveal microscopic crystals. We can surmise that intraerythrocytic Hb C crystal formation occurs in circulating RBC in patients with Hb CC disease, but they are efficiently removed from circulation by the screening function of the spleen.

Another interesting conclusion derived from the study of density gradients is that Hb CC cells are considerably denser than AA cells (Fig. 2). This is in agreement with the well known increase in MCHC described in Hb CC cells (5, 11). It is appropriate to note that RBC

from the splenectomized Hb CC patient have a wider range of density than the RBC from intact homozygous Hb C patients. The wider range of density obscures the reticulocyte band in the splenectomized patient. This suggests that the spleen reduces this range by eliminating the band containing RBC with crystals, and probably other subclasses of erythrocytes. In AA patients, on the other hand, removal of the spleen does not alter the distribution of cells on density gradients. In addition, these gradients demonstrate that Hb CC reticulocytes are considerably denser than normal reticulocytes. This suggests that the increase in MCHC observed in these cells occurs early in the development of these erythrocytes.

NMR linewidths can be used to detect aggregation since when molecular weight increases, the NMR linewidth also increases. The longitudinal and transverse NMR relaxation rates (T_1^{-1} , T_2^{-1}) of nuclei (P-31 in this case) are sensitive to the motion of the nucleus itself and to the motion of the entity in which the nucleus is incorporated. An exact description of relaxation behavior is complexly dependent on both the mechanism of relaxation and the detailed molecular motion (19); however, the generalization can be made that as motion becomes slower T_2 decreases and the linewidth increases. The motion of 2,3-DPG becomes slower when it is incorporated into deoxy hemoglobin. If the deoxy hemoglobin-DPG complex then aggregates we would expect to observe further broadening. Although our approach of using an intracellular effector to monitor aggregation is novel, other investigators have used the transverse relaxation rate of water protons (T_2^{-1}) to monitor hemoglobin S polymerization (20). Sutherland et al. (21), and Noguchi et al. (22) have used C-13 NMR relaxation properties to quantitate polymer in deoxy hemoglobin S solution-gel mixtures.

We have recently reported the use of the T_2 relaxation of P-31 in 2,3-DPG to detect polymerization of hemoglobin S in intact cells (9). The patterns observed upon deoxygenation of hemoglobin C samples are strongly reminiscent of those following deoxygenation of hemoglobin S containing RBC. Our results with Hb C show that following deoxygenation broadening of the 2,3-DPG NMR resonances is considerable and appears as a "hump" replacing or coexisting with the narrower double peaks characteristic of the two phosphates in 2,3-DPG (Fig. 4). This observation is compatible with assigning the broad component of the spectra to aggregates of tetrameric deoxy-Hb C. Furthermore, cells of the splenectomized patient exhibit a larger proportion of aggregated material as a function of deoxy hemoglobin than those of the intact patients. But the most interesting aspect of these results is the demonstration of aggregation of Hb C in whole blood of intact patients. As discussed previously no crystals are detect-

TABLE II
Mean Diameters of Hb AA, Hb CC (Intact), and Hb CC (Splex) Erythrocytes in Whole Blood and Density Gradient Fractions

Blood (Hb)	Whole	Top	Bottom
AA	7.81±0.63	8.11±0.60	7.53±0.53
CC, Intact	6.24±1.25*	7.95±0.77	5.25±0.96*
CC, Splex	6.65±1.15	8.51±0.65†	5.60±0.90*

Values are mean and SD of mean.

* Significant differences as compared to corresponding populations of Hb AA erythrocytes at P value of < 0.001 .

† Significant difference as compared to top fraction of Hb CC (intact) erythrocytes at P value of < 0.01 .

able by Nomarski optics or by scanning electron microscopy in these cells. It should be understood, nevertheless, that the NMR method only detects the deoxy conformer, and it does not imply that only or preferentially this ligand form is capable of aggregation. It does suggest that deoxy-Hb C forms higher molecular aggregates than deoxy-Hb A inside of RBC.

We now turn to the problem of the rheological properties of Hb CC RBC. Neither Murphy (5) nor Self (10) have found the viscosity of Hb CC cells to be ligand-dependant. We have confirmed the increased viscosity of Hb CC cells over a wide range of shear rates and in addition demonstrated that Hb CC cells from splenectomized individuals are more viscous than those from intact patients. This is in perfect agreement with the morphological presence of crystals and the NMR results demonstrating increased aggregation in these cells. Even more interesting, is the finding that Hb CC RBC from the splenectomized patient and to a lesser extent the cells of intact Hb CC patients become more viscous when deoxygenated. This increase in viscosity is significant even when compared with the very large effect seen with SS deoxy cells (Fig. 5). This clearly establishes the ligand dependancy of intracellular aggregation of Hb C and the fact that the deoxy or T conformer of Hb C facilitates the formation of aggregates. The lack of success of other authors (4, 5) in detecting this change is probably derived from the exclusive use of cells from intact patients in which viscosity changes are small.

The high viscosity of RBC from individuals with Hb CC genotype raises the question of their effect on hemodynamics. It is clear that the small pores of the spleen are formidable barriers for these cells and account for the lack of cells circulating with intracellular crystals and the reduction in the range of cell densities detected by the isopycnic gradients in the intact patients. But are these cells in any way onerous to the capillary circulation? We have examined this question with the use of an isolated and artificially perfused rat mesoappendix (Table I). The results conclusively show that no abnormal effect on the microcirculation can be observed with Hb CC cells from intact patients in either the oxygenated or deoxygenated state. In addition, oxygenated RBC from Hb CC splenectomized patient behave quite normally in this physiological preparation. Interestingly, deoxygenated Hb CC RBC from the splenectomized patient have a small but significant effect on the microcirculation. A moderate increase in peripheral resistance and in the pressure flow recovery time were observed. This result is in agreement with the ligand dependancy of the viscosity of these cells and is in further agreement with the interpretation that deoxygenation increases intracellular aggregation. Crystal formation in solutions of Hb C

was also found to be ligand dependant by Charache and co-workers (4).

The lack of abnormal effect of Hb CC cells (from intact patients) on the microcirculation is compatible with the benign clinical course of Hb CC disease even in splenectomized patients and the absence of vascular-occlusive crisis in these patients. Is this result compatible with the higher density of these cells? We have examined this question elsewhere using the perfused rat mesoappendix and comparing AA cells, CC cells of different densities (obtained from density gradients) and rat RBC which are smaller but have the same viscosity as AA cells (11). Further data pertinent to this point is presented in Table II in which diameters of cells are studied. We can conclude that CC cells are not only in average smaller in volume (4, 11) but are clearly smaller in diameter, particularly in the intact patient. In the splenectomized patients the average of RBC diameters is closer to normal suggesting, but not proving, that the spleen plays a role in the reduction of cell diameter in Hb CC cells. The fact that Hb CC cells are smaller in volume and diameter than normal cells allows for their normal capillary transit, and compensates for their increased viscosity in terms of their hemodynamic impact.

In summary, Hb CC cells can have crystals intracellularly when circulating, but these are efficiently removed by the spleen. Hb CC also undergoes intracellular aggregation quite readily even when no crystals can be detected. This process is clearly favored by deoxygenation. Hb CC cells are normal or near normal with respect to their hemorheological properties by virtue of the smaller size, which compensates for their increased viscosity. The process of reduction in size and increase in density begins early in the development of the Hb CC erythrocyte and, as it is present in the splenectomized individual, the mechanism involved might not necessitate the presence of the spleen.

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REFERENCES

1. Itano, H. A., and J. V. Neel. 1950. New inherited abnormality of hemoglobin. *Proc. Natl. Acad. Sci. U. S. A.* **36**: 613-617.
2. Spaet, T. H., R. H. Alway, and G. Ward. 1953. Homozygote type "C" hemoglobin. *Pediatrics* **12**: 483-490.
3. Levin, W. C., R. G. Schneider, J. A. Cudd, and J. E. Johnson. 1953. A family with homozygous hemoglobin C and sickle cell trait union. A clinical hematological

- and electrophoretic study. *J. Lab. Clin. Med.* **42**: 918. (Abstr.)
4. Charache, S., C. L. Cauley, D. F. Waugh, R. J. Ugoretz, and J. R. Spurrell. 1967. Pathogenesis of hemolytic anemia in homozygous hemoglobin C disease. *J. Clin. Invest.* **46**: 1795–1811.
 5. Murphy, J. R. 1968. Hemoglobin CC disease: Rheological properties of erythrocytes and abnormalities in cell water. *J. Clin. Invest.* **47**: 1483–1495.
 6. Lessin, L. S., W. N. Jensen, and E. Pondée. 1969. Molecular mechanism of hemolytic anemia in homozygous hemoglobin C disease: Electron microscopic study by the freeze-etching technique. *J. Exp. Med.* **130**: 443–466.
 7. Kraus, A. P., and L. W. Diggs. 1956. *In vitro* crystallization of hemoglobin occurring in citrated blood from patients with hemoglobin C. *J. Lab. Clin. Med.* **47**: 700–705.
 8. Wheby, M. S., O. A. Thorup, and B. S. Leavell. 1956. Homozygous hemoglobin C disease in siblings: further comment on intraerythrocytic crystals. *Blood*. **11**: 266–272.
 9. Fabry, M. E. 1980. Detection of Hemoglobin S Polymerization in Intact Red Cells by P-31 NMR. *BBRC*. **97**: 1399–1406.
 10. Self, F., L. V. McIntire, and B. Zanger. 1977. Rheological evaluation of hemoglobin S and hemoglobin C hemoglobinopathies. *J. Lab. Clin. Med.* **89**: 488–497.
 11. Kaul, D. K., S. Baez, and R. L. Nagel. 1981. Flow properties of oxygenated Hb S and Hb C erythrocytes in the isolated microvasculature of the rat: a contribution to the hemorheology and hemoglobinopathies. *Clin. Hemorrh.* Vol. 1, pp. 73–86, 1981.
 12. Vettore, L., L. S. Azzolina, and M. C. DeMattis. The Application of a New Density Gradient System to the Separation of Blood Cells. XVII Congress of the International Society of Hematology. July 1978, Paris, France.
 13. Opella, S. J., D. J., Nelson, and O. Jardetzky. 1976. Dynamics of Nuclear Overhauser enhancement in proton decoupled carbon-13 magnetic resonance. *J. Chem. Phys.* **64**: 2533–2536.
 14. Baez, S., H. Lamport, and A. Baez. 1960. Pressure effects in living microscopic vessels. In *Flow Properties of Blood and Other Biological Systems*. A. Copley, and G. Stainby, editors. Pergamon Press, London 122–136.
 15. Green, H. D., C. E. Rapela, and M. C. Conrad. 1963. Resistance (conductance) and capacitance phenomena in terminal vascular beds. *Handb. Physiol.* **2** (Section 2, Circulation): 935–960.
 16. Baez, S. 1973. A method for on-line measurement of lumen and wall of microscopic vessels *in vivo*. *Microvasc. Res.* **5**: 299–308.
 17. Gupta, R. K., J. L. Benovic, and Z. B. Rose. 1979. Location of the allosteric site for 2,3-diphosphoglycerate on human oxy- and deoxy-hemoglobin as observed by magnetic resonance spectroscopy. *J. Biol. Chem.* **254**: 8250–8255.
 18. Schmid-Schonbein, H., R. E. Wells, and J. Goldstone. 1971. Fluid drop-like behavior of erythrocytes. Disturbances in pathology and its quantification. *Biorheology*. **7**: 227–234.
 19. Deslauriers, R., and I. C. P. Smith. 1980. Biological Magnetic Resonance. Vol. 2. L. J. Berliner and J. Reuben, editors. Plenum Publishing Corp., New York.
 20. Cottam, G. L., M. R. Waterman, and B. C. Thompson. 1977. Kinetics of polymerization of deoxyhemoglobin S and mixtures of hemoglobin A and hemoglobin S at high hemoglobin concentrations. *Arch. Biochem. Biophys.* **181**: 61–65.
 21. Sutherland, J. W. H., W. Egan, A. N. Schechter, and D. A. Torchia. 1979. Carbon-13-proton nuclear magnetic double-resonance study of deoxyhemoglobin S gelation. *Biochemistry*. **18**: 1797–1803.
 22. Noguchi, C. T., D. A. Torchia, and A. N. Schechter. 1979. C-13 NMR quantitation of polymer in deoxyhemoglobin S gels. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 4936–4940.