Compound Heterozygosity for Hemoglobin and Korle-Bu: Moderate Microcytic Hemolytic Anemia and Acceleration of Crystal Formation

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We report here that compound heterozygosity for hemoglobin Korle-Bu (HbKB) and HbC (β6 Glu → Lys) is associated with moderate chronic hemolytic anemia with microcytosis. To understand the pathogenesis of this syndrome, we have studied the effect of Hb Korle-Bu (KB = β73 Asp → Asn) on the crystallization of HbC. We have previously established that fetal Hb (HbF) inhibits the crystallization of HbC. In contrast, HbF accelerates crystallization affecting the pathogenesis of SC disease. We now report on in vitro crystallization of mixtures of HbKB, HbC, and various amounts of HbF and the native hemolysate of a child who is a compound heterozygote for HbKB and HbC. At 6 months of age, the propositus' hemolysate contained 55% HbKB, 39% HbC, and 6% HbF. Crystals formed within 2 minutes compared with 30 minutes for the mixture of 40% HbC:60% HbF and with 180 minutes for 40% HbC:60% HbA. The morphology of the crystals formed was cubic, in contrast with the tetragonal crystals observed in CC and SC disease. Early crystals did not exhibit "sharp edges" until 45 minutes. Purified HbKB formed aggregates but not crystals after 24 hours. Isopycnic gradients showed that the KB/C compound heterozygotes have red blood cell (RBC) densities intermediate between the AC and CC phenotype and similar to SC disease. The surface residue β73, known to participate in areas of interaction of the deoxy HbS polymer, can now be assigned to areas of contact in HbC containing crystals. The hemolysis observed in the HbKB/C compound heterozygote is likely to be secondary to the acceleration of HbC crystallization. The microcytosis and increased RBC density is clearly the consequence of the presence of HbC, but the basis of the increased RBC pathology compared with AC trait, despite the low proportion of HbC (35% to 40%), remains to be elucidated.

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THE HOMOZYGOS STATE for hemoglobin C (HbC) (CC disease) is characterized by the tendency to form intraerythrocytic crystals.1 Intraerythrocytic HbC crystals in circulating red blood cells (RBCs) are in the liganded state (oxygenated), and cells containing intraerythrocytic HbC crystals have no detectable fetal Hb (HbF).2 In vitro studies of the kinetics of crystallization of HbF mixed with HbC show the inhibition of HbC crystallization by HbF. Moreover, by comparison with the inhibitory effect of several selected Hbs, we concluded that residue β87 Glu is at least partially the cause,3 making this residue a likely contact site in the oxy HbC crystal.

When compound heterozygosity for abnormal Hbs occurs, the mutants have the potential of interacting with each other, generating a phenotype different from that of each component. SC disease (compound heterozygote for HbS and HbC) is a case in point in which the properties of HbC (reduction in cell size and increase in MCHC) and the presence of 50% HbS generate a syndrome that is intermediate between AS or AC and SS.4

More recent studies show that HbS accelerates HbC crystallization (via an alternate mechanism that involves co-crystallization with HbC), implying that crystallization in addition to polymerization may play a role in SC disease.5 As previously suspected by Diggs et al,1 tetragonal crystals have been found in circulating erythrocytes from SC patients.6

Microcytosis with elevated MCHC (density) is a characteristic of HbC containing RBCs.7 The mechanism of these phenomena have been thought by some to be related to the increased activity of K:Cl cotransport.8 Nevertheless, CC cells lack sufficient K:Cl activity to explain the RBC density differences with SS cells, and their permanently reduced volume was an enigma until recently. Altered regulation of the K:Cl cotransport activity in CC cells (particularly their reduced capacity to turn off the transporter) may explain their permanent reduction in mean corpuscular volume (MCV) and increase in MCHC.9

Here, we report an infant with compound heterozygosity for HbC (β6 Glu → Lys) and Hb Korle-Bu (HbKB; β73 Asp → Asn) presenting with moderate, chronic hemolytic anemia and microcytic hyperdense RBCs. To understand the pathogenesis, we investigated the kinetics of crystallization of hemolysates obtained from the infant (and purified forms of these Hbs) as well as the behavior of these cells in isopycnic gradients.

MATERIALS AND METHODS

Clinical data. KM was born to an 18-year-old primipara after 9 months of gestation, characterized by excessive bleeding at 5.5 months and blunt abdominal trauma shortly preterm. The hyperactive and labile mother attempted suicide twice (at 6 months with an overdose of vitamins and at 7 months with 4 Diabinese pills) and abused illicit drugs during pregnancy. The baby had a normal birth weight but had a facial palsy and feeding abnormalities that required further evaluation. At 1 month of age, the infant was admitted with metabolic acidosis, which resolved, but opisthotonos and paresis or palsy of cranial nerves V, VII, VIII, and IX were noted. These deficits were accompanied by anisocoria, facial asymmetry, exposure keratitis, and a marked developmental lag. Brain stem...
auditory-evoked response was markedly abnormal. Chromosome analysis was normal, and lymphoid hyperplasia was noted on bowel survey. The diagnosis of Moebius syndrome \(^{11}\) was made. Testing for a viral etiology was negative for toxoplasmosis, syphilis, cytomegalovirus, herpes simplex, and human immunodeficiency virus (HIV).

Anemia was noted at 10 weeks of age, with an Hb of 9 g/dL (Table 1). The smear showed anisocytosis, many target cells, and some schistocytes. Hb electrophoresis showed that one-quarter was HbF, almost half was HbG (later identified as HbKB), and the rest HbC. In the next 3 years, the infant had Hb levels between 9.8 and 10.4 g/dL, low MCVs between 68 and 76 fL, and reticulocyte counts between 2.4% and 3.7%, with occasional bouts of higher reticulocytemia as high as 2.4%.

*Hb analysis.* Hemolysates were prepared by freeze-thawing erythrocytes with subsequent centrifugation to remove membranes. Through a combination of cellulose acetate (pH 8.6) and agar citrate electrophoresis (pH 6.4), it was established that the propositus had HbC and a major Hb migrating in the position of HbG, in addition to variable amounts of HbF (depending on the age of the infant). The Hb migrating in the position of HbG was isolated and purified from the mother’s (heterozygote for HbG and HbA) hemolysate and identified as HhKB.

Quantification of the components present in a hemolysate was performed with the use of a densitometer (Beckman scanner; Beckman, Fullerton, CA) with clarified cellulose acetate anion exchange liquid chromatography with gel (DE-52) and identification of the HbG as HhKB. High performance liquid chromatography (HPLC) separations of globin chains and tryptic digests were performed on Vydac C-4 and C-18 reverse phase columns (Separations Group, Hesperia, CA), respectively, and developed with acetonitrile:water/TFA gradient. The mutation was determined by mass spectrometry, as described in detail elsewhere. \(^{12}\) In brief, molecular masses of globins precipitated from abnormal Hb were found to be within 1 d range from normal values, as determined by electrospray mass spectrometry. The abnormal \(\beta\) globin isolated by reversed phase HPLC was digested with trypsin, and the resulting peptides were separated using a C-18 HPLC column. The \(\beta\)T9 peptide migrated faster than normal, and its molecular mass was found to be 1 d less than normal (MH’ 1,668.9 d). Of three possible one-point mutations resulting in the found molecular mass, the presence of the \(\beta\)T9 Asp \(\rightarrow\) Asn substitution, specific for the \(\beta\)Kfree-\(\beta\)K, was unequivocally proved by tandem mass spectrometry sequencing.

Cells from whole blood were separated on a Percoll-Larex (equivalent to Stractan) density gradient as described by Fabry et al. \(^{13}\)

Kinetics of crystal formation and morphologic analysis of crystals. Crystals were generated under the conditions described by Adachi and Asakura \(^{14}\) by incubating purified Hb(s) (2 g/100 mL) in high concentration phosphate buffer, pH 7.4, 1.8 mol/L at 30°C. \(^{15}\) Aliquots obtained after specific time intervals were removed from the incubating solution, and crystal counts were made using a Zeiss light microscope with the aid of a hematocytometer.

**RESULTS**

Electrophoretic separation of Hbs in the propositus hemolysate and identification of HbKB. In Fig 1, the starch gel electrophoresis of the hemolysate from the 3-month-old in-

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**Table 1. Hemoglobin Composition and Hematology of Propositus by Age**

<table>
<thead>
<tr>
<th>Age</th>
<th>%Hb Composition</th>
<th>Hematologic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HbKB</td>
<td>HbC</td>
</tr>
<tr>
<td>7 d</td>
<td>Hbs F, G, and C</td>
<td>15.6</td>
</tr>
<tr>
<td>10 wk</td>
<td>48.5</td>
<td>27.4</td>
</tr>
<tr>
<td>6 mo</td>
<td>55.0</td>
<td>39.6</td>
</tr>
<tr>
<td>1.5 yr</td>
<td>57.8</td>
<td>39.1</td>
</tr>
<tr>
<td>3 yr</td>
<td>64.0</td>
<td>34.9</td>
</tr>
</tbody>
</table>

At 7 days, no quantification, but in order of band intensities.

Abbreviations: HCT, hematocrit; Retics, reticulocytes.
Crystal kinetics. In vitro crystals did not form in the 10-week-old infant’s hemolysate containing 24.1% HbF (Table 1), consistent with the observation that HbF inhibits crystal formation. In contrast, the 6-month-old infant’s hemolysate (Table 1) showed precrystal structures within the resolution time of the observation (which is less than 2 minutes; Fig 3). Precrystal structures (Fig 4A) formed in a greatly accelerated manner compared with the controls (100% HbC and mixtures of 40% HbC and 60% HbS) and occurred either with or without a minimal delay or lag phase (Fig 3). The corresponding control, mixtures of 40% HbC and 60% HbA formed tetragonal crystals in 3 hours (data not shown), whereas the mixtures of 40% HbC and 60% HbS formed tetragonal crystals in 30 minutes, and with 100% HbC tetragonal crystals formed by 15 minutes (Figs 3 and 4).

Crystal morphology. The precrystalline structures observed almost immediately after the propositus’ hemolysate at 6 months of age (Table 1) were incubated in the high-concentration phosphate buffer as shown in Fig 4. The edges of the precrystal structures (Fig 4A) appear less sharp than the morphologically distinct cubic crystals that emerged after about 60 minutes of incubation (Fig 4B). Nevertheless, these are of a different crystal habit than the typical tetragonal crystals that form with 100% HbC or with the control mixtures containing HbC but not HbKB (Fig 4C). Exposure of the KB/C blood to 3% NaCl results in the generation of crystals in some of the cells, but the edges of these intraerythrocytic bodies are not as sharp as in CC disease blood.

Hb composition of the crystals. We generated crystals with the hemolysate of the propositus at age 18 months, which had a composition of Hbs as follows: HbKB 57.8%, HbC 39.1%, and HbF 3.1% (Table 1). The crystal analysis showed HbKB of 40%, HbC of 60%, and 0% HbF. Hence, the cubic crystals observed in vitro have a different composition than the hemolysate favoring the presence of HbC and excluding HbF.

RBC morphology. We examined smears of the propositus at different ages. Although the smear was clearly abnormal with poikilocytosis and a dramatic increase in “folded cells” similar to those observed in CC and SC disease (Fig 5), we did not observe clear intracellular crystals with sharp edges, even in the most dense RBCs isolated from density gradients. Although some cells were suspected of having small or middle size “bodies” intracellularly (Fig 5C), none were the typical one or two intraerythrocytic crystal type that consume all the RBC Hb observed in splenectomized CC patients (Fig 5C) and in SC patients.

### Table 2. RBC Density Distributions of KBC, CC, SC, AC, and AA Genotypes

<table>
<thead>
<tr>
<th>Age</th>
<th>KBC</th>
<th>CC</th>
<th>SC</th>
<th>AC</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mo</td>
<td>3.0</td>
<td>0.2</td>
<td>1.9</td>
<td>5.5</td>
<td>4.6</td>
</tr>
<tr>
<td>3 yr</td>
<td>51.6</td>
<td>31.3</td>
<td>31.1</td>
<td>68.2</td>
<td>75.8</td>
</tr>
<tr>
<td>9 yr</td>
<td>25.8</td>
<td>40.0</td>
<td>40.8</td>
<td>17.9</td>
<td>15.4</td>
</tr>
<tr>
<td>15 yr</td>
<td>19.5</td>
<td>28.5</td>
<td>26.2</td>
<td>8.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Abbreviations: D1, MCHC < 33 g/dL or d < 1.076; D2, 33 < MCHC < 37 g/dL or 1.076 < d < 1.091; D3, 37 < MCHC < 42 g/dL or 1.091 < d < 1.110; D4, MCHC > 42 g/dL or d > 1.110.
DISCUSSION

We report here that double heterozygosity for HbKB and HbC generates a syndrome characterized by moderate, chronic hemolytic anemia and microcytosis. Because each of the heterozygous forms of this genotype (namely AC and AKB traits) are not associated with disease, it is highly probable that the interaction between HbKB and HbC by a mechanism similar to the pathogenic interaction between HbC and HbS found in SC disease is the cause of the abnormalities. To define the type of interactions involved, we studied the kinetics of crystal formation.

The data presented here show that HbC, in the presence of HbKB, exhibits accelerated crystal formation, and crystallization occurs either with or without a minimal delay or lag phase (Fig 3). In addition, the final cubic crystal (different in morphology from the tetragonal HbC crystals) is preceded by high-order aggregates without sharp edges (Fig 4A). This latter observation suggests that mechanisms of crystal growth (and maybe nucleation) are different for HbC in the presence of HbKB, resulting in a different crystal structure.

The profound effect of HbKB (β73Asp → Asn) on oxy HbC crystal formation implies that β73 is a contact site in the crystal and that the disappearance of a negatively charged group changes the microenvironment of β73 and defines a different crystallization pathway. The slight oxygen equilibrium difference observed in HbKB compared with HbA is also compatible with this interpretation. It is of interest to note that β73 forms part of the lateral contact site in the double strand of the deoxy HbS crystal, is active in solution as shown by binary Hb mixtures, and explains the diminished capacity for polymerization of HbC Harlem.
Fig 5. Propositus’ RBC morphology by scanning electron microscopy. (A) The erythrocytes from the Percoll-Larax middle fraction appear abnormal; stomatocytes, “pancake” shaped RBCs, and tridimpelled cells are observed (original magnification × 2,000). (B) The RBCs in the densest Percoll-Larax fraction appear the most abnormal with many “folded” cells (original magnification × 3,000). (C) Occasionally, RBCs with intracellular bodies are observed in the densest fraction (original magnification × 10,000).

(β6 Glu → Val; β73Asp → Asn), which has one mutation identical to HbS (β6Val) and another, in the same β chain, identical to HbKB.29

In complete accord with these findings are the studies of Adachi and Asakura,29 who confirmed that HbC Harlem had decreased polymerization25 and discovered that, in addition, this doubly mutated Hb has an increased tendency to crystallize. This last property can be assigned to the presence of the β73 mutation, because β8 containing Hbs do not crystallize under these conditions.

The lack of morphologically apparent crystals (defined as sharp edged intracellular bodies) in KB/C RBCs is puzzling at first sight. Because the initial crystals are not sharp edged (Fig 4A), the only bodies that should be expected intracellularly are the ones depicted in Fig 5C. The final cubic crystals might not have a chance to form in vivo if most of the precrystal containing RBCs are removed by the spleen.

Although these experiments do not definitively prove that increased crystallization is the cause of hemolysis in KB/C disease, they suggest that crystal formation is a leading candidate.

The other feature of this syndrome is the presence of microcytosis with RBC hyperdensity (and hyperdense reticulocytes). Isopycnic gradient separation shows not only that the KB/C RBCs are small, as the MCV shows, but also that their density distribution is broader than the densities of AC or CC RBCs and is similar to SC RBCs (Fig 1).46 The increase in MCHC could involve mechanisms similar to those described for CC, that is, increased activity and abnormal regulation of the K:Cl cotransport.9,10 The presence of dense reticulocytes observed by density gradient centrifugation, very reminiscent of the phenomenon observed in CC and SC disease, supports this hypothesis. The exact mechanism(s) involved and the role of HbKB in exaggerating the density effect of 40% HbC remain to be defined. Nevertheless, at a minimum, the increased RBC destruction observed in this case should increase the proportion of young cells that have a higher expression of the K:Cl cotransport.22,23

A case of HbKB and HbC double heterozygosity in an adult with a phenotype of moderate hemolytic anemia and microcytosis has recently been reported in abstract form,24 confirming the results presented here, and partially reported in a previous abstract.25

In summary, the combination of HbKB and HbC produces a mild microcytic hemolytic syndrome and shows in vitro acceleration of crystal formation in which precrystal Hb structures convert rapidly into classical crystals. In the presence of HbKB, cubic-type crystal formation occurs instead of the tetragonal crystal structure of CC, SC, and AC. The residue β73, located on the surface, must be involved (directly or indirectly) in intermolecular contacts of the Hbc/KB crystal. The microcytosis and increased density of KB/C RBCs, dense reticulocytes, and “folded RBCs” are the consequence of the presence of Hbc but are amplified by the presence of HbKB by a mechanism yet to be defined.

We conclude that KB/C double heterozygosity is a hemoglobinopathy with many of the qualitative characteristics of HbC heterozygosity, but made quantitatively more intense by the presence of Hb KB. The relatively high frequency of HbKB (G Accra) and HbC among African-Americans suggest that this combination will be observed clinically with some frequency.

REFERENCES
Compound heterozygosity for hemoglobin C and Korle-Bu: moderate microcytic hemolytic anemia and acceleration of crystal formation [corrected] [published erratum appears in Blood 1994 May 15;83(10):3105]

RL Nagel, MJ Lin, HE Witkowska, ME Fabry, M Bestak and RE Hirsch