Hemoglobin Köln in a Black: Pre- and Post-Splenectomy Red Cell Survival (DF\(^{32}\)P and \(^{51}\)Cr) and the Pathogenesis of Hemoglobin Instability


A 17-yr-old black male with hemolysis and pigmenturia but no anemia was found to have hemoglobin Köln (\(\alpha_2\beta_2\) Val\(^{-}\)-Met\(^{1.9}\)) \((a2\beta2\) Val\(^{-}\)-Met\(^{1.9}\)) [FGS]). Splenectomy was done because of complicating thrombocytopenia. Thrombo-kinetic studies with \(^{51}\)Cr tagged platelets suggested hypersplenism, and after surgery the platelet count returned to normal. The red cell \(T_{1/2}^{51}\)Cr was more than doubled, but the red cell life span (DF\(^{32}\)P) was more modestly improved (30.6 \(\rightarrow\) 47.2 days). The “elution” of \(^{51}\)Cr from the red cells presplenectomy was 5.6%/day, whereas after surgery it was normal (1.9%/day), accounting for the disparity between the survival methods. Study of the isolated cyanferri derivative of hemoglobin Köln by ultracentrifugation at various salt concentrations and various pH's indicated an increased tendency to dimer formation under conditions where normal hemoglobin is a tetramer. This results from the site and type of amino acid substitution and accounts in part for its instability.

HEMOGLOBIN KÖLN DISEASE was first described in 1962, but the structural abnormality of the variant was not determined until 1966. Since that time over 20 cases from several families have been reported, making hemoglobin Köln probably the most common unstable hemoglobin variant associated with hemolytic disease and red blood cell inclusion bodies. Individuals with this disorder have thus far been mostly of English and German ancestry. It is our purpose to report the first black with hemoglobin Köln disease, along with red cell survival studies with DF\(^{32}\)P and \(^{51}\)Cr and evidence concerning the chemistry of instability.

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Table 1. Clinical Laboratory Data.

<table>
<thead>
<tr>
<th></th>
<th>Presplenectomy</th>
<th>Postsplenectomy</th>
<th>Normal</th>
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<tr>
<td>Hemoglobin (gm/ml)</td>
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<td>14.4</td>
<td></td>
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<tr>
<td>Hematocrit (ml/100 ml)</td>
<td>51.0</td>
<td>52.5</td>
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<tr>
<td>Red cell count (x 10^6/cu mm)</td>
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<td>4.71</td>
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<td>Platelet count (x 10^3/cu mm)</td>
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<td>White cell count (x 10^9/cu mm)</td>
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<td>4.6-5.5</td>
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<td>Reticulocyte count (%)</td>
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<td>Bilirubin (direct/total; mg%)</td>
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<td>0-2</td>
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<tr>
<td>Plasma hemoglobin (mg/100 ml)</td>
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<td>1.3</td>
<td>0-2</td>
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<tr>
<td>Serum haptoglobin (mg/100 ml)</td>
<td>0</td>
<td>0</td>
<td>50-150</td>
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<tr>
<td>Heinz bodies (fresh blood)</td>
<td>4+</td>
<td>4+</td>
<td>0</td>
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<tr>
<td>Red cell 2.3 DPG (m moles/L)</td>
<td>4.5</td>
<td>5.3 ± 0.1</td>
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<td>Red cell G-6-PD (U/gm Hb)</td>
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<td>Red cell pyruvate kinase (U/gm Hb)</td>
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<td>Methemoglobin (%)</td>
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MATERIALS AND METHODS

Routine hematologic studies were made using standard procedures. Red cell metabolic studies were done by methods referenced in Table 1. Other studies included flow characteristics of the red cells through polycarbonate sieves, red blood cell autosurvival with both the 51Cr and DF32 P methods before and after splenectomy, and platelet kinetics with 51Cr pre- and postsplenectomy. Blood was collected from the patient prior to splenectomy, mixed with ACD solution, and sent packed in ice by air mail special delivery from Washington, D.C. to Augusta, Ga. Red cell hemolysate was prepared immediately upon arrival. The saline washed cells were lysed with an equal volume of distilled water. Stroma was removed by shaking with 0.2 volume of carbon tetrachloride for 1 min and centrifuging for 15 min at 10,000 g, and at 4°C.

Starch gel electrophoresis of hemoglobin was carried out at pH 9.0. The alakali-resistant hemoglobin was determined according to the method of Betke, Marti, and Schlicht. The heat stability of the abnormal hemoglobin fraction present in the hemolysate was determined by a modification of the method of Grimes, Meisler, and Dacie.

The abnormal hemoglobin fraction was isolated by chromatography on columns of DEAE-Sephadex equilibrated in 0.04 M Tris-HCl buffer, pH 8.0. The fraction containing the abnormal hemoglobin was concentrated on a small column of CM-Sephadex, pH 6.5. Elution from CM-Sephadex was made with 0.2 M Tris-HCl buffer, pH 8.5. The eluate was dialyzed against distilled water for 24 hr, and converted into globin using the acid-acetone method. Structural analyses used procedures outlined previously.

Sedimentation velocity analyses followed procedures described in previous studies, using a Spinco model E analytical ultracentrifuge at a rotor speed of 60,000 rpm, and a temperature of 25°C. Hemoglobin concentrations were 0.5 g/100 ml. The cyanferri derivative was used because of its greater stability. It was prepared by treating the fractions isolated from a DEAE-Sephadex column with Drabkin's solution. The excess ferricyanide was removed by dialysis against distilled water. The ratios of the optical densities of the isolated hemoglobin A and hemoglobin KÖln at 540 and 280 nm and at 415 and 280 nm were the same. This observation excludes a significant decrease in heme content of the isolated cyanferri hemoglobin KÖln. The following buffers were used at approximately 0.1 ionic strength: citric acid-Na2HPO4 (pH range, 4.9-5.9); KH2PO4-NaOH (pH range, 4.9-5.9); NaCl-glycine-NaOH (pH range, 4.9-10.7); and Na2HPO4-NaOH (pH range, 4.9). Each sample was dialyzed for 24 hr at 4°C against three changes of the appropriate buffer. The sedimentation coefficients were calculated from the peak positions in the schlieren patterns and corrected to 20°C in water in the usual manner. The partial specific volume (f) of the hemoglobins was assumed to be 0.749 ml/g at 20°C and 0.751 ml/g at 25°C.
Case Report

In 1965, a 17-yr-old black male was admitted to the District of Columbia General Hospital for evaluation of dark urine which dated to early childhood. There was no other history of illness. He had an elevated reticulocyte count, but no anemia (Table 1). A peripheral blood smear showed mild red cell anisocytosis and hypochromia with occasional macrocytes, target cells, and spherocytes. Diffuse and punctate basophilia were present. Platelets were slightly decreased but had normal morphology, as did white blood cells. Heat-precipitable hemoglobin was present. Although no Heinz bodies were found in fresh red cells, they did appear following incubation with acetylphenylhydrazine. At the time of a flight physical examination in military service in November 1969, dark urine, minimal scleral icterus, and hepatosplenomegaly prompted further evaluation at Walter Reed General Hospital (WRGH). Thrombocytopenia, previously mild, was now moderately severe. A bone marrow aspirate was hypercellular with normoblastic erythroid hyperplasia (E:M ratio of 1:1). The number of megakaryocytes was increased, granulopoiesis appeared normal, and iron stores were mildly decreased. The results of $^{51}$Cr tagged platelet studies (Table 2) suggested "hypersplenism," and in August 1970 a spleen weighing 840 g was removed. The histopathology was that of congestive splenomegaly with a loss of white pulp markings and a thickened capsule. There was marked sinusoidal and cord congestion with prominent reactive germinal centers. Recovery from surgery was uncomplicated and thrombocytopenia was no longer present. Heinz bodies were now found in nearly every red cell. The peripheral smear showed changes consistent with the postsplenectomy state and hemolysis. Platelets appeared normal in number and had normal morphology except for a few moderately large ones.
Miscellaneous Laboratory Studies

Urine studies for hemoglobin, myoglobin, porphobilinogen, reducing substances (Clinitest), and protein were negative. The visible light absorption spectrum for the urine was nonspecific and similar to that described by others. Hemoglobin was present in the urine. Although initially bile was present in the urine, in later studies it was always absent. Liver biopsy was normal. Coombs tests were negative; serum glutamic-oxaloacetic transaminase, lactic dehydrogenase, alkaline phosphatase, protein electrophoresis, uric acid, urea nitrogen, creatinine (and creatinine clearance), calcium, phosphorus, electrolytes, and amylase were all normal, as were prothrombin time, partial thromboplastin time, fibrinogen level, and thrombin time.

Erythrokinetic Studies

Before splenectomy, the plasma $^{59}$Fe clearance was rapid, and the plasma iron turnover increased. The peak red blood cell $^{59}$Fe utilization was 65$^{\circ}/_{\text{o}}$, or slightly less than normally found. Although there was some improvement in true red cell life span after splenectomy ($^{32}$P 30.6 → 47.2 days), it was not as great as suggested by the results of the $^{51}$Cr studies ($^{32}$P 30.6 → 47.2 days). The “elution rate” (Ke), i.e., the difference between loss of circulating $^{32}$P and circulating $^{51}$Cr, was 5.6$^{\circ}/_{\text{o}}$/day presplenectomy but only 1.9$^{\circ}/_{\text{o}}$/day after surgery. Before the spleen was removed, there was increased accumulation of $^{51}$Cr activity over the spleen as determined by external counting. RBC mass was slightly above normal (35 ml/kg body weight) before splenectomy and was essentially unchanged after surgery.

Enthrocute Metabolic Evaluation

The results of various studies are comparable to those found in a young red cell population (Table 1). The ascorbic acid-cyanide screening test for G-6-PD deficiency was abnormal despite normal activity of that enzyme by assay. This test is known to be a valuable screening test comparable in sensitivity and lack of specificity to the measurement of autohemolysis. Blood containing an unstable hemoglobin has been found to give an abnormal test.

After recovery from splenectomy, his red cells passed normally through a 5-$\mu$ pore size polycarbonate sieve. On the other hand, there was reduced flow through 3-$\mu$ pores (60$^{\circ}/_{\text{o}}$ of normal).

Hemoglobin Studies

Starch gel electrophoresis of hemoglobin in hemolysate from blood shipped to Augusta, Ga., showed an abnormal band which migrated slightly slower than hemoglobin S (Fig. 1). The DEAE-Sephadex chromatogram of this hemolysate showed two major and three minor zones (Fig. 2). The major hemoglobin Köln zone and hemoglobin A$_2$ were incompletely separated. A third (minor) fraction was eluted between hemoglobin Köln and hemoglobin A; this hemoglobin was later identified also as hemoglobin Köln. This incomplete separation was overcome on preparative columns by using columns of DEAE-Sephadex equilibrated with 0.04 M Tris-HCl pH 8.0. About 10$^{\circ}/_{\text{o}}$–12$^{\circ}/_{\text{o}}$ of the
component was present in fresh hemolysate; the electrophoretic and chromatographic mobilities of this fraction changed considerably upon aging, which made quantitation difficult.

The heat stability curve of hemoglobin in this hemolysate is presented in Fig. 3. Approximately 20% of the hemoglobin was precipitated within 10 min after incubation at 65°C, as compared with about 3% of the normal control.

**Structural Studies**

Amino acid analyses of 72 hr hydrolysates of the β chain gave these results (values are mean of five separate analyses; data between parentheses represent the theoretical values for the normal β chain): Lys 10.9 (11); His 8.9 (9); Arg 3.1 (3); Asp 13.3 (13); Thr 6.5 (7); Ser 4.6 (5); Glu 11.1 (11); Pro 6.9 (7); Gly 12.6

**Fig. 2.** Elution profile of hemoglobin of red cell hemolysate from subject N. L. on a column (0.9 x 50 cm) of DEAE-Sephadex A-50. The chromatogram was developed with 0.05 M Tris-HCl buffers. The broken line represents the pH of the effluent.
Fig. 3. The instability of the hemoglobin of red cell hemolysate from subject N. L. on warming at 65°.

These data indicate that the abnormality concerns the probable replacement of a valyl residue by a methionyl residue. All peptides were recovered from the tryptic digest of the AE-β-Köln chain except T-12b; the purity of T-10 did not allow an accurate analysis of this peptide. The amino acid composition of all other peptides is similar to those of the corresponding peptides of the β chain of the normal hemoglobin A except that of β T-11. This peptide did not contain the expected valyl residue but did contain instead a methionyl residue. (Composition: His 0.90, Arg 1.00; Asp 2.10; Glu 1.18; Pro 0.86; Met 1.01; Leu 0.83; Phe 0.95.) This indicates a replacement of the valyl residue in position 98 (FG5) of the β chain by a methionyl residue as has been observed in the β chain of the hemoglobin Köln.2

The differences in the sedimentation properties of the cyanferri derivatives of hemoglobins A and Köln are shown in Fig. 4, which compares the respective S_{20,w} values in NaCl solutions of increasing molarity. These values were calculated from the observed sedimentation coefficients at 25°C (S_{obs}) using a value of 0.751 ml/g for the partial specific volume (φ) of hemoglobin at 25°C at all concentrations of NaCl.29 Correction of the observed sedimentation coefficients in this manner does not take into consideration the effects of preferential solvent interactions, i.e., binding of salt or water by protein.29,33 Failure to consider such interactions at high salt concentrations results in sedimentation coefficients which are lower than the true value if water is preferentially bound: such theoretical considerations do not, however, affect comparisons of the behavior of normal and mutant hemoglobins.

In 0.1 M NaCl near neutral pH the S_{20,w} value of cyanferrihemoglobin Köln was 4.38 S, which is only slightly lower than the value of 4.48 S observed for
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Fig. 5. The dependence of the sedimentation coefficients ($S_{20,w}$) of the cyanferri derivatives of hemoglobins Köln and A upon pH at 25°C. o, hemoglobin A; e, hemoglobin Köln.

cyanferrihemoglobin A (Fig. 4). When the concentration of NaCl was increased to 0.25 M, $S_{20,w}$ of hemoglobin Köln dropped to a value of 3.71 S, whereas that of hemoglobin A remained unchanged. The sedimentation coefficients of hemoglobin Köln decreased further with increasing salt concentration; in 2 M NaCl $S_{20,w}$ was 2.83 S, compared to 3.83 for hemoglobin A.

Figure 5 compares the sedimentation properties of cyanferrihemoglobin Köln and A as a function of pH. Maximum $S_{20,w}$ values of 4.2 S to 4.4 S were observed for hemoglobin Köln between pH 7.4 and pH 8.5. Below pH 6 and above pH 9, the marked decrease in sedimentation coefficient indicates that liganded hemoglobin Köln is extensively dissociated into dimers under conditions where hemoglobin A is a tetramer. In pH 10.1 buffer, for example, hemoglobin Köln is almost completely dimeric ($S_{20,w} = 2.95$ S) in contrast to hemoglobin A, which is still predominantly tetrameric ($S_{20,w} = 4.26$ S). At pH 10.7 dissociation into dimers appears to be complete for both hemoglobins.

Family Studies

A paternal uncle, the propositus’ brother, and the propositus’ son were studied. None had pigmenturia, anemia, or elevation of the reticulocyte count. Red cells from each formed a few Heinz bodies after 24-48 hr incubation at 37°C, but hemoglobin Köln could not be demonstrated by heat stability and starch gel electrophoretic analyses.

DISCUSSION

Heinz bodies are denatured or denaturing globin which stains with certain supravital dyes such as brilliant cresyl blue. Amino acid substitutions or deletions affecting one of the heme attachment sites or the sites of bonds controlling the tertiary structure of the molecule may result in an unstable hemoglobin. The molecular and clinical aspects of unstable hemoglobin syndromes have recently been reviewed. Heinz bodies may also form when there are certain enzyme abnormalities, such as G-6-PD deficiency, which adversely affect the ability of the red cell to withstand oxidative stress.

Our propositus has a history rather common for a patient with an unstable hemoglobin, complaining only of dark urine since infancy. The range of symptoms mentioned in earlier cases extends from pigmenturia alone or in association with mild fatigue to a moderately severe nonspherocytic hemolytic anemia. Some patients have also developed complicating thrombocytopenia. Early laboratory investigations suggested increased red blood cell turnover in
our patient. Our structural analyses leave no doubt that the heat labile abnormal variant is identical to hemoglobin Köln in which a methionyl residue replaces the valyl residue at the position 98 of the β chain.2

Recent studies by Wajcman et al.35 have shown that when hemoglobin Köln is in the oxygenated conformation, the molecule is saturated with heme groups and stable. Our analyses indicate that cyanferri hemoglobin Köln is also heme-saturated. Transition to the deoxygenated state results in loss of the heme group from the β chain with subsequent instability, precipitation, and Heinz body formation. The ability of the oxy derivative to form a stable, heme-saturated molecule, in contrast to that of the deoxy form, is likely related to the fact that position β 98 is in a region that is directly involved in the conformational transition from the oxygenated to the deoxygenated state, and is also itself directly involved in this transition.36,37

The sedimentation velocity properties of hemoglobin Köln indicate that the cyanferri derivative (and presumably other liganded forms) has an increased tendency to dissociate into dimers, in agreement with earlier data obtained by differential gel filtration.38 Displacement of the tetramer-dimer equilibrium towards the dimeric form has been observed for the liganded derivatives of a number of abnormal hemoglobins.19,26,27,28,39,40 In contrast, the unliganded derivatives of these mutants (those which remain stable upon deoxygenation) have been found to retain a predominantly tetrameric structure, similar to deoxyhemoglobin A, even at moderately high salt concentrations.28,39,40 This tendency to dissociate in one form, but not in the other, is likely due to the conformational differences which exist between the oxy and deoxy structures and to the stabilizing effect of the six additional salt bonds which are present in the deoxygenated molecule.36,37

Most of the hemoglobin variants which exhibit increased dimer formation in the liganded form have an amino acid substitution in one of the positions that make up the α1β2 contact, or in close proximity to such a position. Hemoglobin Köln is no exception. In normal oxyhemoglobin, valine in position β 98 (FG5) forms such a contact with threonine in position α1 (C6).36,41,42 In addition, position β 98 is in the region of several other α1β2 contacts in both the oxy and deoxy conformations.36,37,41 Thus, it is possible that replacement of the valyl residue by the larger methionyl residue may cause disruption not only of the contact involving β 98, but of the adjacent ones as well.

The presence of hemoglobin Köln in a black subject is noteworthy because it has previously been reported predominantly in persons of northern European ancestry.11 Our findings and those of Lie-Injo et al., who reported its presence in a Malay family,10 suggest that this hemoglobinopathy may be more widely distributed. Studies of three family members failed to establish direct genetic transmission. Potentially significant is a northern European white ancestor four generations removed from the propositus.

The most commonly used technic for measuring red cell auto-survival involves tagging with 51Cr.43 As chromate, this ion passes the red cell membrane where it is reduced to Cr3+ and attaches to hemoglobin, predominantly at the β chain.44 Recently, significant improvement in the red cell 51Cr T1/2 was reported following splenectomy in a patient with hemoglobin Köln disease.5 The obser-
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...vations in our subject show a similar improvement. On the other hand, a more modest improvement was seen in the red cell survival when measured with DF-32P. The difference can be explained by a more rapid removal of the 51Cr label from red cells when the spleen is intact because the Heinz bodies containing the unstable protein are readily "pitted" from the red cells by the spleen.45 Moreover, the hemoglobin Köln β chains are said to bind 3–7 times more 51Cr than do normal ones.11 An increase in splenic radioactivity detected by body surface scanning is usually interpreted as splenic sequestration or destruction of red cells.4647 Some consider this an indication for splenectomy.47 Splenic pitting of tagged inclusions would give the same picture, as it did for our subject in the studies done in 1966. Consequently, it was not considered an indication for splenectomy, which was done later because of thrombocytopenic hypersplenism. We are unaware of similar combined tag studies in patients with unstable hemoglobins or Heinz body anemias.

In a recent review, note was taken of the disparity between the apparent rate of hemolysis and the lack of anemia in patients with hemoglobin Köln disease.11 One reason is over estimation of the hemolytic rate with the 51Cr technic as discussed above. Additionally, hemoglobin Köln has been found to have an increased affinity for oxygen,48 thus releasing less oxygen to tissues and stimulating erythropoiesis. Our patient had a high hemoglobin level and a mild increase of red cell mass. His bone marrow was readily able to compensate for the three- to fourfold increase in red cell destruction to produce the mild "polycythemia" secondary to the abnormality in the oxygen dissociation curve.

Others have reported thrombocytopenia in patients with hemoglobin Köln.3 The moderately severe depression of the platelet count in our patient was likely due to splenic sequestration of platelets, as implied by the platelet kinetic studies. Poor presplenectomy 1 hr recovery of tagged platelets (13%), normal presplenectomy platelet life span, normal 1 hr recovery postsplenectomy (97%), and normalization of the platelet count after splenectomy confirm that thrombocytopenia in hemoglobin Köln disease results from hypersplenism.

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