Hemoglobin Köln Disease Occurring as a Fresh Mutation: Erythrocyte Metabolism and Survival

By Denis R. Miller, Robert I. Weed, George Stamatoyanopoulos, and Akira Yoshida

Electrophoretic and fingerprinting studies in a patient with congenital hemolytic anemia revealed the presence of the unstable hemoglobin Köln (β98 val→met). Examination of parents and siblings gave normal results. Extensive blood group and isozyme studies were consistent with the thesis that Hb Köln disease in the propositus was the result of a fresh mutation in one of his parent's gametes. In the propositus, the activities of enzymes of the Embden-Meyerhof and pentose phosphate pathways were increased, but the level of ATP was decreased. Methemoglobin reduction was delayed when the NADPH-dependent system was utilized with added methylene blue and gave a false-positive result in the glucose-6-phosphate dehydrogenase screening test. Methemoglobin reduction in the absence of methylene blue was normal. Increased methemoglobin and Heinz body formation, decreased osmotic fragility, decreased red cell deformability, and a disproportionate potassium loss without sodium gain occurred with metabolic depletion. The rate of decline of glutathione in propositus' cells paralleled that in normal cells. Autologous survival of Hb Köln cells was decreased but was not compromised further by oxidant drugs. Marked splenic sequestration of Hb Köln erythrocytes was demonstrated, and an excellent response to splenectomy with improved erythrocyte survival was observed. The intracellular precipitation of unstable globin chains, intracellular dehydration, and increased membrane rigidity probably all contribute to the splenic entrapment of these erythrocytes.

Structural alterations of human hemoglobin producing increased lability of hemoglobin chains1,2 result in a hemolytic syndrome defined as unstable hemoglobin disease. Hb Köln (β98 val→met)3 is one of more than 20 from the Department of Pediatrics, Division of Pediatric Hematology, Cornell University Medical College, New York, N.Y., the Departments of Pediatrics and Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N.Y., and the Department of Medicine, Division of Medical Genetics, University of Washington School of Medicine, Seattle, Wash.

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abnormal hemoglobins associated with molecular instability. It is also the most common, having been described in four unrelated families.\textsuperscript{3-7} We present data on another family with Hb Köln in which both parents of the propositus are clinically normal and lack an unstable hemoglobin in their red cells. Blood group and isozyme data in this family make it very probable that Hb Köln disease appeared in the propositus as a fresh mutation affecting the $\beta_{98}$ structural site of the hemoglobin chain. In addition to the hemoglobin and family data, studies of red cell metabolism and survival in the propositus are presented.

**MATERIALS AND METHODS**

Standard hematologic studies were performed by conventional methods.\textsuperscript{8} Measurements of glucose utilization, lactate production, adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3-DPG),\textsuperscript{9} assays of Embden-Meyerhof and pentose phosphate shunt enzymes,\textsuperscript{10} and glutathione (GSH) and GSH stability\textsuperscript{11} were performed by the methods cited. Methemoglobin and methemoglobin reductase were quantified by the method of Beutler and Baluda.\textsuperscript{12} Autohemolysis and osmotic fragility of defibrinated blood were determined by the methods of Young and co-workers.\textsuperscript{13} Activities of erythrocyte GSH reductase,\textsuperscript{14} NADPH methemoglobin reductase,\textsuperscript{15} acetylcholinesterase,\textsuperscript{16} and glutamic-oxalacetic transaminase (GOT)\textsuperscript{17} were performed by methods previously published.

Survival and organ sequestration of erythrocytes labeled with sodium chromate ($\text{Na}_2^{51}\text{CrO}_4$) were performed as described by Jandl and co-workers.\textsuperscript{19} The effect of oxidant drugs on survival was also measured. The propositus was given sulfisoxazole, 60 mg/kg per day during an autologous study, and in a cross-transfusion study the patient’s informed mother was given his compatible, labeled cells. Forty-eight hours later, primaquine base, 15 mg daily, was started, and erythrocyte survival and organ sequestration were measured. Spleen localization index was calculated according to described methods.\textsuperscript{20} Plasma clearance of $^{59}$Fe was measured by the method of Dacie and Lewis.\textsuperscript{8} Intracellular monovalent cation (Na$^+$ and K$^+$) concentration was measured by flame photometry as described by Weed and Bowdler,\textsuperscript{21} and active potassium influx was measured by the method of Funder and Wieth.\textsuperscript{22} Erythrocyte deformability was measured by the method of La Celle.\textsuperscript{23}

A heat stability test was used to demonstrate hemoglobin instability.\textsuperscript{24} Heinz body formation and hemoglobin autoxidation were studied in red cells incubated under sterile conditions, following the technique of Huehns and co-workers.\textsuperscript{25} Other hemoglobin studies included: horizontal starch gel electrophoresis using a Tris-EDTA-borate buffer, pH 8.6; DEAE-Sephadex chromatography using a Tris-$\text{HCl}$ 0.01 M buffer with a linear pH 8.1-pH 7.0 gradient; and CM-Sephadex chromatography using phosphate buffer pH 6.0 with a 0.05–0.1 M elution gradient. Globin was prepared with acid acetone precipitation, and hemoglobin chains were separated by the method of Clegg et al.\textsuperscript{26} Peptide chains were S-aminoethylated, digested by trypsin,\textsuperscript{27} and fingerprinted by the method of Baglioni.\textsuperscript{28} Peptide spots were stained by ninhydrin and platinum chloride reagent.\textsuperscript{29} Amino acid analysis was performed using an automatic amino acid analyzer.

**CASE REPORT**

The propositus is a 13-yr-old boy who was admitted at 9 yrs of age (May 1967) because of recurrent jaundice. During early childhood he had frequent upper respiratory tract infections and asthmatic bronchitis associated with pallor and dark urine. In 1962, at 4 yrs of age, he was treated with oral ferrous sulfate because of anemia. In 1967, weakness, anorexia, scleral icterus, light stools, and dark urine were noted after an episode of bronchitis. A diagnosis of infectious hepatitis was made, and improvement occurred in 4 wk. Three months later, again following bronchitis, he complained of fatigue, cola-colored urine, and jaundice. The heterophile agglutination test was negative, SGOT 30 U, and total
HEMOGLOBIN KÖLN DISEASE

bilirubin 1.8 mg/100 ml. Because of persistent jaundice and splenomegaly, he was referred for diagnostic evaluation.

His weight was at the 50th and his height at the 97th percentile. Pertinent findings included icteric sclerae, posterior cervical and axillary adenopathy, and splenomegaly (4 cm below the left costal margin).

Splenectomy was performed in June 1969. The spleen weighed 380 g. Heinz bodies were seen in a spleen imprint preparation with phase contrast and electron microscopy (Fig. 1). Following the operation, the patient was markedly improved symptomatically, and the color of his urine was lighter.

RESULTS

Hematologic Findings

Standard studies are listed in Table 1. The persplenectomy peripheral smear showed frequent polychromatophilia, moderate anisocytosis, occasional poikilocytosis and microspherocytosis, and rare punctate basophilia. The bone marrow aspirate was hypercellular with marked normoblastic erythroid hyperplasia. The serum iron was 182 µg/100 ml, and the iron binding capacity 354 µg/100 ml. The serum folate and B₁₂ levels were 28 ng/ml and 310 pg/ml.

Other Laboratory Findings

Tests for urinary coproporphyrin and porphobilinogen were negative. The urobilinogen titer was 1:32, and the Perl's stain for hemosiderin was strongly positive. The SGOT, alkaline phosphatase, bromosulfalcin retention, prothrombin time, total protein and albumin, protein electrophoresis, BUN, uric acid, creatinine, and electrolytes were normal.

Demonstration of Hemoglobin Instability

Heinz bodies were not identified after incubation of the propositus' blood for 30-120 min but were noted in every cell after 24-48-hr incubation at 37°C. Heating of the hemolysates for 10 min at 50°C revealed a fine precipitate of hemoglobin that was not present in hemolysates containing only hemoglobin A. More prolonged incubations resulted in the formation of a flocculent red-white precipitate.

Hemoglobin Studies

On electrophoresis, a smeared hemoglobin fraction migrating near the position of Hb F, (Fig. 2) was noted. Other gels showed a fraction migrating with the rate of free alpha chains. The findings of free alpha chains in the absence of a double dose of beta thalassemia suggested that the propositus was a carrier of an unstable β-chain variant of hemoglobin. In some electrophoretic preparations, instead of one, three hemoglobin bands were observed in the early development of the stain, a major one migrating with the rate of Hb F and two other bands with migration intermediate between that of the major fraction and of Hb A₂. The proportions of the three fractions were estimated as 5%, 2%, and 1%, respectively. Three abnormal fractions were also eluted by DEAE- or CM-Sephadex chromatography. Fetal (alkali-resistant) hemoglobin was not elevated, and Hb A₂ was within the normal range.

Amino acid analysis of purified β chain of the chromatographically pure
Fig. 1.—Electron photomicrograph of spleen from propositus. Erythrocytes (R) are in passage through cord (C) and between sinus littoral cells (L) into splenic sinus (S). Cordal macrophages (CM) are loaded with inclusions or Heinz bodies (H) of varying size, and one contains a phagocytosed erythrocyte (R₁). Single inclusion body is seen in sinus littoral cell. (Pb citrate and uranyl acetate stain.) × 10,000.

major fraction contained two methionine residues per molecule, in contrast to one methionine per one normal \( \beta \) chain. The pattern of peptide mapping of \( \beta \) chain under study was identical to that of normal \( \beta \) chain. The platinum reagent, specific for sulfur containing amino acids, gave a positive reaction in
HEMOGLOBIN KÖLN DISEASE

Table 1.—Hematologic Values in Propositus and His Parents

<table>
<thead>
<tr>
<th></th>
<th>Propositus</th>
<th>After Splenectomy</th>
<th>Father</th>
<th>Mother</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>38</td>
<td>48</td>
<td>48</td>
<td>41.5</td>
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<tr>
<td>Hemoglobin (g/100 ml)</td>
<td>10.5</td>
<td>14.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Red cell count (x10^6/cu mm)</td>
<td>4.18</td>
<td>5.08</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MCV (cu μ)</td>
<td>90.9</td>
<td>94.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MCH (μg)</td>
<td>25.1</td>
<td>27.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>28.3</td>
<td>29.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Reticulocyte count (%)</td>
<td>7-16</td>
<td>3.7</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Absolute reticulocytes (per cu mm)</td>
<td>292-668,000</td>
<td>187,000</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Platelet count (per/cu mm)</td>
<td>177,000</td>
<td>318,000</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Bone marrow (M:E ratio)</td>
<td>1:3.58</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Heinz body preparation*</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Heinz body preparation†</td>
<td>Positive</td>
<td>—</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Coombs’ test (γ and non-γ)</td>
<td>Negative</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ham’s test</td>
<td>Negative</td>
<td>Negative</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Haptoglobin (mg/100 ml)</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Total heme pigment (mg/100 ml) (plasma)</td>
<td>41.3</td>
<td>—</td>
<td>—</td>
<td>4.4</td>
</tr>
<tr>
<td>Methemalbumin (mg/100 ml) (plasma)</td>
<td>41.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hb Köln (%)†</td>
<td>8.0</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Performed in fresh blood; † performed in blood incubated for 24–48 hr at 37°C. Test is considered negative when less than 5% of cells contain Heinz bodies; † by DEAE Sephadex chromatography.

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

the peptide spot β T-11 of the abnormal hemoglobin. Normal β T-11 was negative. Amino acid analysis of β T-11 peptides from the abnormal hemoglobin revealed that one valine residue was missing and a methionine residue appeared. On the basis of the established sequence of beta chain, this finding corresponds to substitution of β 98 valine by methionine, the amino acid substitution found in Hb Köln.*

Fig. 2.—Starch gel electrophoretic pattern of propositus, parents, and normal hemolysates. Hb Köln migrates cathodal to Hb A but is “smeared.” Free alpha chains migrate cathodally to origin and are not seen in this gel. (TRIS-EDTA-Borate pH 8.6.)
**Table 2.—Erythrocyte Metabolic Studies**

<table>
<thead>
<tr>
<th>Pentose Phosphate Shunt and Methemoglobin Reduction</th>
<th>Before Splenectomy</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD screening test Abnormal</td>
<td>3.02</td>
<td>1.57±0.2</td>
</tr>
<tr>
<td>G6PD *</td>
<td>1.78</td>
<td>1.00±0.2</td>
</tr>
<tr>
<td>G6PD (mg/100 ml RBC)</td>
<td>68.5</td>
<td>60–90</td>
</tr>
<tr>
<td>GSH stability (% change)</td>
<td>+1.6</td>
<td>±10</td>
</tr>
<tr>
<td>GSH reductase*</td>
<td>1.47</td>
<td>1.2–2.0</td>
</tr>
<tr>
<td>NADPH Met-Hb reductase*</td>
<td>1.80</td>
<td>2.5–3.5</td>
</tr>
<tr>
<td>H₂O₂ hemolysis (%)</td>
<td>10.0</td>
<td>0–10</td>
</tr>
<tr>
<td>Methemoglobin (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh blood</td>
<td>0.45</td>
<td>1.0</td>
</tr>
<tr>
<td>Brewer’s test †</td>
<td>69.0</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>Blood incubated for 48 hr</td>
<td>49.0</td>
<td>3.5±1.0</td>
</tr>
<tr>
<td>With glucose</td>
<td>19.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**Embden-Meyerhoff Pathway**

| Glucose utilization (μmoles/ml RBC per hr) | 3.45 | 2.13±0.09 |
| Per cent reduction with ouabain (0.1mM)    | 23   | 10       |
| Lactate production (μmoles/ml RBC)         | 7.65 | 4.19±1.17 |
| ATP (μmoles/ml RBC)                       | 0.905| 1.410±1.17 |
| ATP Stability (per cent change after 4 hr) | −7.3 | ±10     |
| 2,3 DPG (μmoles/ml RBC)                   | 5.22 | 4.19±0.2 |
| Hexokinase*                              | 0.39 | 0.18±0.1 |
| G3PD *                                   | 19.5 | 17.3±6.5 |
| PK *                                     | 5.95 | 1.65±0.12 |

* Activity expressed in U/10¹⁰ red blood cells.
† Incubation with sodium nitrate, glucose, and methylene blue for 3 hr.

**Erythrocyte Metabolic Studies**

The activities of selected enzymes of the pentose phosphate shunt and Embden-Meyerhof pathway, included in Table 2, were elevated and reflected the young mean cell age. The activity of GSH reductase and the GSH content were normal; GSH was stable following 2 hr of incubation with acetylphenylhydrazine (APH). With prolonged (8–24 hr) incubation in the presence and absence of APH, the content of GSH decreased at the same rate in the normal and patient’s cells. With sterile 24–48-hr incubations in the presence or absence of glucose but without APH, GSH declined more rapidly during the first 24 hr in the patient’s cells but the GSH content was similar in the patient and the control cells at 48 hr.

There was normal methemoglobin concentration in fresh blood, but delayed methemoglobin reduction was noted in cells treated with sodium nitrite and incubated with glucose, sodium nitrite, and methylene blue. After 3 hr, 69% of the patient’s hemoglobin was in the oxidized form, whereas the level in normal blood was 1%. These quantitative studies of methemoglobin reduction confirmed the consistently abnormal results obtained in the screening test in which glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes are unable to reduce nitrite-induced methemoglobin. Methemoglobin reduction progressed normally after washing the cells repeatedly to remove sodium.
The activity of NADPH methemoglobin reductase was slightly decreased when the patient's methemoglobin was used as substrate.

Methemoglobin formation was markedly increased in the patient's cells after 48 hr of incubation with saline or with nutrient additives, when compared to normal cells. Glucose inhibited methemoglobin formation to a greater degree than adenosine > inosine > saline (Fig. 3).

Glucose utilization and lactate production were increased and reflected the young red cell population. Although the concentration of ATP was low (0.905 μmoles/ml RBC), the ATP was stable after 4 and 6 hr of incubation. The content of 2,3-DPG was raised (5.22 μmoles/ml RBC).

Autohemolysis of untreated and methemoglobinized blood was normal. Osmotic fragility and intracellular monovalent cation content were determined after 24 hr of sterile incubation in tubes to which were added either normal saline, glucose (20 mM), adenosine (30 mM), or inosine (30 mM). When compared to fresh blood, the osmotic fragility was most decreased with saline <glucose < adenosine < inosine (Fig. 4). The alterations of intracellular cation concentration followed the changes in the osmotic fragility curves (Table 3). A large K⁺-loss, a small Na⁺-gain, and a large net total cation loss were noted with normal saline. Potassium loss was minimal with inosine and adenosine and slight with glucose, and a slight Na⁺-gain similar to that without nutrient additives occurred. The marked reduction (77%) in intracellular K⁺ and disproportionately small increment in Na⁺ accompanied the markedly decreased osmotic fragility with normal saline. The progressive normalization of the osmotic fragility curves after incubation in glucose, adenosine, and inosine
are reflected by the greater stability of the intracellular K+ content. The intracellular monovalent cation content of fresh propositus cells was normal. A slight increment in Na+, a slight decrease in K+, and a slight increase in total monovalent cation content occurred in normal cells studied similarly.

These studies were repeated after splenectomy. Compared to preoperative values, sodium gain was increased with the addition of saline or ouabain (0.1 mM), and potassium loss was decreased in the presence of glucose. Inhibition of active cation flux with ouabain resulted in a net loss of cation (as K+) and decreased osmotic fragility.

Active potassium influx was increased (propositus, 2.84 mM/liter RBC per hr; control, 1.87). Ouabain-sensitive K influx was 1.76 mM/liter RBC per hr (control, 1.59), and ouabain-insensitive K influx was increased (1.08 mM/liter RBC per hr vs. 0.30 in control cells). Potassium efflux (leak) was 2.5-fold normal.

The deformability of propositus cells (presplenectomy) was decreased. The

Table 3.—Intracellular Monovalent Cation Content

<table>
<thead>
<tr>
<th></th>
<th>Fresh Blood</th>
<th>24-hr Incubation</th>
<th>NaCl (pre)</th>
<th>Glucose (pre)</th>
<th>Adenosine (pre)</th>
<th>Inosine (pre)</th>
<th>Ouabain (post)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(pre)</td>
<td>(post)</td>
<td>(moles x 10^-15/RBC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propositus</td>
<td>Na^+</td>
<td>0.4</td>
<td>2.9</td>
<td>1.2</td>
<td>2.1</td>
<td>1.3</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>K^+</td>
<td>9.0</td>
<td>2.1</td>
<td>2.5</td>
<td>7.0</td>
<td>3.0</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>Na^+ + K^+</td>
<td>9.4</td>
<td>3.8</td>
<td>5.4</td>
<td>11.3</td>
<td>9.6</td>
<td>6.6</td>
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<tr>
<td>Normal</td>
<td>Na^+</td>
<td>0.9</td>
<td>2.8</td>
<td>—</td>
<td>1.3</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>K^+</td>
<td>9.2</td>
<td>8.1</td>
<td>—</td>
<td>8.6</td>
<td>8.2</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>Na^+ + K^+</td>
<td>10.1</td>
<td>10.9</td>
<td>—</td>
<td>9.9</td>
<td>9.6</td>
<td>11.3</td>
</tr>
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</table>

* Presplenectomy.
† Postsplenectomy.
‡ Na^+; intracellular sodium.
§ K^+; intracellular potassium.
Table 4.—Red Blood Cell Survival and Organ Sequestration Studies

<table>
<thead>
<tr>
<th>Study</th>
<th>RBC Survival (days $\frac{1}{2}$)</th>
<th>Organ Sequestration Ratio (maximal)</th>
<th>Spleen Localization Index</th>
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<tbody>
<tr>
<td>Autologous, presplenectomy</td>
<td>7.5</td>
<td>3.1 5.1</td>
<td>2.1 4.7</td>
</tr>
<tr>
<td>Autologous + sulfisoxazole</td>
<td>7.0</td>
<td>4.6 2.6</td>
<td>2.0 4.7</td>
</tr>
<tr>
<td>Cross-transfusion + primaquine</td>
<td>4.0</td>
<td>2.1 2.6</td>
<td>1.6 6.1</td>
</tr>
<tr>
<td>Autologous, postsplenectomy</td>
<td>17.6</td>
<td>— — 1.4</td>
<td>— —</td>
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<tr>
<td>Normal Values</td>
<td>26–38</td>
<td>1.0 1.0</td>
<td>1.0 1.0</td>
</tr>
</tbody>
</table>

* S:L, spleen:liver.
† S:H, spleen:heart.
‡ L:H, liver:heart.

RBC Survival and Organ Sequestration Studies

The half-life ($t\frac{1}{2}$) of $^{51}$Cr-labeled autologous erythrocytes was 7.5 days with marked splenic sequestration, as demonstrated by the organ ratios and by calculation of the spleen localization index. The $t\frac{1}{2}$ of autologous erythrocytes during treatment with sulfisoxazole was the same (7.0 days). When the patient's cells were given to the mother whose red cells do not contain abnormal hemoglobin the $t\frac{1}{2}$ was 4.0 days. Splenic sequestration was demonstrated, but there was no difference in the survival curve during the control period or during primaquine ingestion (Table 4).

Erythrocyte survival 6 mo after splenectomy revealed a $t\frac{1}{2}$ of 17.6 days.

Family Study

The patient's parents are in good health; the mother is of English and the father is of German ancestry. There was no consanguinity, nor was the mother exposed to radiation, toxins, infections, or other known mutagenic agents. No family history of blood dyscrasias, anemia, gall bladder disease, or splenectomy was obtained. Hematologic findings were normal in the parents (Table 1) and in the two healthy male siblings. Abnormal hemoglobin fractions on electrophoresis or chromatography, heat-precipitable hemoglobin, increased methemoglobin, or Heinz body formation with incubation were not detected in the parents or the siblings. These data indicate that neither the parents nor the siblings was carrier of Hb Köln.

In order to test paternity, blood groups, serum factors, and red cell isozymes were examined. The results (Tables 5 and 6) make it very unlikely that the propositus is the offspring of an illegitimate mating.

Discussion

Hemoglobin Köln disease is a compensated hemolytic anemia characterized by recurrent episodes of jaundice, dark urine ("dipyrroluria"), and frequently, splenomegaly. The hemolytic syndrome typically manifests in the heterozygotes
<table>
<thead>
<tr>
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<th>Rh</th>
<th>Kell</th>
<th>Duffy</th>
<th>Kidd</th>
<th>Lewis</th>
<th>P</th>
<th>MNS</th>
<th>Lutheran</th>
<th>Diego</th>
<th>Sex</th>
<th>Linked</th>
<th>G/C</th>
<th>Serum Groups</th>
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<td>Father</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2-1</td>
</tr>
</tbody>
</table>
HEMOGLOBIN KÖLN DISEASE

Table 6.—Red Cell Isozyme Phenotypes in Propositus and Parents

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Father</th>
<th>Mother</th>
<th>Propositus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Phosphatase</td>
<td>BA</td>
<td>BA</td>
<td>B</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>2</td>
<td>2–1</td>
<td>2–1</td>
</tr>
<tr>
<td>Phosphohexose isomerase</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NADH diaphorase</td>
<td>usual</td>
<td>usual</td>
<td>usual</td>
</tr>
<tr>
<td>Adenosine deaminase</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Peptidase A</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Peptidase B</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

and consequently presents with an autosomal dominant mode of inheritance, although sporadic cases do occur. In our case, studies of the parents and siblings of the propositus failed to show evidence of an unstable hemoglobin. Other examples of suspected fresh mutations have been encountered in the two cases with Hb Hammersmith, in Hb-Ube-1, in Hb Freiburg, and in several other occasions where unstable Hb disease is probable from the clinical picture. In contrast to mutations that do not affect hemoglobin function (and thus pass unnoticed), mutations causing hemoglobin instability are clinically ascertained because of the resultant hemolytic syndrome. The recording of such cases will ultimately provide better estimates of the mutation rates in man. On the basis of data with Hb Hammersmith, a mutation rate of $1 \times 10^{-4}$ per nucleotide or $1 \times 10^{-3}$ per gene has been proposed.

The discovery of hemoglobins associated with instability and those associated with polycythemia has stressed the importance of testing hemoglobin function in the diagnosis and management of syndromes caused by abnormal hemoglobins. The oxygen dissociation curve of the Hb Köln is shifted to the left of normal, and the P50 O2 (partial pressure of oxygen when hemoglobin is 50% saturated) of Hb Köln is low. The expected consequences of increased oxygen affinity of Hb Köln and the leftward shift of the oxygen dissociation curve would be decreased delivery of oxygen to the tissues, increased erythropoietin secretion, and increased erythrocyte production. This sequence of events in our patient is supported by the presence of a well-compensated hemolytic anemia prior to splenectomy and by the normal values for hemoglobin and packed cell volume obtained following removal of the spleen.

Oxygen affinity of red cells is also influenced by the intracellular organic phosphates, 2,3-DPG and ATP, that act as allosteric competitors to oxygen and modify the hemoglobin-oxygen equilibrium. These compounds bind reversibly with deoxyhemoglobin, resulting in a shift of the oxygen dissociation curve to the right. The concentrations of 2,3-DPG and ATP have not been reported in other documented cases of Hb Köln disease, although Lelong and co-workers noted decreased ATP and 2,3-DPG in a patient with congenital hemolytic anemia and pigmenturia. Although the level of 2,3-DPG was increased in the erythrocytes of our patient, the concentration of ATP was decreased, and thus the total content of 2,3-DPG and ATP was less than ex-
pected for the degree of reticulocytosis. Increased ATP utilization in monovalent cation pumping could contribute further to the decreased levels of ATP, although ATP was stable during a 6 hr-incubation period. Furthermore, ATP may be irreversibly bound to precipitated Hb Köln. The location of the molecular defect in β 98, a residue of importance for chain-chain interaction, is probably one adequate cause for the abnormal O₂ affinity in Hb Köln. However, a combined effect due to the amino acid substitution and also to abnormal binding of the two allosteric compounds to Hb Köln cannot be excluded, but clarification of the mechanism of increased oxygen affinity must await further studies. Wajcman and colleagues recently reported that 2,3-DPG binds to stripped Hb Köln but does not modify its oxygen affinity.

In the red cell containing Hb Köln, several secondary metabolic abnormalities occur and are of importance in determining its fate. Increased GSH utilization, decreased levels of erythrocyte GSH, and GSH instability have already been documented. Jacob et al. found excessive GSH binding in mixed disulfide linkage to the cysteine in the 93rd position of the β chains of Hb Köln. They also noted increased K⁺ loss, Na⁺ accumulation, osmotic fragility, and autohemolysis when membrane sulfhydryl groups were inhibited by paramercuribenzoate. Under different experimental conditions, we found that the formation of methemoglobin and Heinz bodies increased, and the levels of GSH decreased with glucose deprivation. However, decreased total intracellular monovalent cation content and, concomitantly, decreased osmotic fragility occurred. The loss of potassium exceeded the gain in sodium and resulted in intracellular dehydration that has been observed in other congenital hemolytic anemias.

When quantified by heat denaturation or electrophoresis, Hb Köln accounted for approximately 10%–20% of the total hemoglobin in most previous reports. White and Brain recently presented evidence for a deficiency in the synthesis of βKöln-chains, rather than increased precipitation of denatured Hb Köln, to explain the seemingly low concentration of Hb Köln in heterozygotes. Despite only 8% Hb Köln in our patient, approximately 50% of his hemoglobin was methemoglobin after prolonged incubation in the absence of glucose. This might occur if dimers of αβKöln containing ferrihemes interacted with dimers of HbA, resulting in a tetrameric hybrid methemoglobin molecule α₂βKölnₙₙ¢α₂βₐ, or if ferrihemes dissociating from Hb Köln exchanged with heme of HbA.

It has been proposed that intraerythrocytic Heinz body formation in unstable hemoglobin diseases is caused by the dissociation of heme from globin with precipitation of insoluble, heme-deficient β chains. The increased membrane rigidity and the resultant decreased erythrocyte deformability enhances splenic sequestration of Hb Köln erythrocytes. These cells are dependent upon glucose to maintain normal levels of ATP and GSH, intracellular cation homeostasis, and cell shape. Thus the static, acidotic, and hypoxic environment of the splenic microcirculation would be detrimental to these cells. Cinephotomicrographic and electron micrographic demonstration of splenic en-
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Phenotypic variation observed in our patient was anticipated, although not all investigators have shown that splenectomy is beneficial.32

Unstable hemoglobins are of pharmacogenetic importance. This susceptibility of the abnormal pigments to autoxidation and intraerythrocytic precipitation and the decreased levels of reduced glutathione in these red cells provide ideal conditions for the potentiation of hemolysis by oxidant drugs. Drug-induced hemolysis has been observed with Hb Zürich,45 with Hb H disease, with Hb Torino, and possibly, in a case of Hb Köln.46 In our case, no adverse effects on cell survival were evident with the administration of either sulfisoxazole to the patient or primaquine to his mother who had received a transfusion of her son’s cells. In another patient with Hb Köln disease,6 phenacetin had no measurable effect upon the already shortened survival of Hb Köln cells. In spite of these negative results, administration of potentially hemolytic drugs to a patient with unstable hemoglobin should be undertaken with caution, since differences between individuals in the metabolism of the oxidant drugs may be of importance in determining the potentially harmful levels of noxious metabolites. Thus, a failure to detect a hemolytic effect of sulfonamides or primaquine in one family with Hb Köln disease may not apply in other cases with the same defect.

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HEMOGLOBIN KÖLN DISEASE


Hemoglobin Köln Disease Occurring as a Fresh Mutation: Erythrocyte Metabolism and Survival

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