Hereditary, X-Linked, Sideroachrestic Anemia. The Isolation of Two Erythrocyte Populations Differing in Xg\(^a\) Blood Type and Porphyrin Content

By G. R. Lee, W. D. MacDiarmid, G. E. Cartwright and M. M. Wintrobe

Severe hypochromic anemia was observed in male members of a family reported by Cooley\(^1\) and later by Rundles and Falls (family II).\(^2\) In these families, female carriers were not anemic, but a small population of abnormal, hypochromic erythrocytes were seen in smears of their blood. Similar families have been reported by others,\(^3\) and in some instances the affected subjects have improved with pyridoxine therapy. The genetic data in some of these families have suggested an X-linked hereditary pattern in which the gene has incompletely recessive characteristics.

One explanation for the coexistence of two morphologically distinct erythrocyte populations in the carrier female members of the above families is provided by the Lyon hypothesis.\(^4\) This holds that in all cells of normal females, one or the other of the X chromosomes is genetically inactive. By this mechanism, an X-linked defect could lead to two cell lines, one in which the active X chromosome contains the normal gene and one in which the active X chromosome carries the abnormal gene.

A possible tool for investigating the above hypothesis is provided by the discovery of a human, X-linked erythrocyte antigen, the Xg\(^a\) blood group.\(^5\) The usefulness of this antigen is limited by the weakness of its reactivity as compared with that of other blood group antigens. Gorman and co-workers were unsuccessful in their attempt to utilize the Xg\(^a\) blood group to establish mosaicism in female carriers of glucose-6-phosphate dehydrogenase deficiency.\(^6\)

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Further study led them to suggest that X-inactivation did not apply to the Xg* locus.

We have studied a family, three female members of which were found to have a population of hypochromic, microcytic erythrocytes mingled with normal cells. The low density of the abnormal cells enabled us to separate them from the normal cells by physical means. In the two affected individuals who were heterozygous for the Xg* antigen, the isolated erythrocyte populations differed in Xg* blood type.

Isolated cell populations also were studied with respect to porphyrin content and synthesis. The abnormal population contained reduced amounts of protoporphyrin, suggesting a heme-biosynthetic defect. A preliminary report of this work was published elsewhere.7

**METHODS**

Methods used for routine hematologic measurements are those described by Cartwright.8 Technics used in studying red cell porphyrin content and porphyrin biosynthesis also are given elsewhere.9 Methods used for the determination of serum iron10 total iron-binding capacity,11 red cell glutathione,12 glucose-6-phosphate dehydrogenase,13 ferrokinetics,14 and chromosomal karyotype15 have been described by others.

Erythrocyte were separated according to density by centrifugation in layered gum acacia solutions.16 The lower gum acacia layer was adjusted to a specific gravity of 1.060 and the upper to 1.050. Blood diluted fourfold with normal saline was layered on top of the gum acacia and the mixture centrifuged at 4500 × g for 20 min.

Initial tests for the Xga antigen were kindly performed by Dr. Joseph D. Mann. Subsequent studies were performed by the Coombs blood-grouping technic5 with antiserum supplied by Dr. Mann. In all cases, an albumin control and a panel of cells of known Xga reactivity were examined simultaneously.

**Case Report**

V.N., a Caucasian female of German and English ancestry, was first seen in 1956, at the age of 6. Mild anemia had been observed six months previously in the course of an investigation of a respiratory infection; however, no data were available to exclude an earlier onset. When no response to iron-containing medications given either by mouth or parenterally, was observed, the patient was referred to this clinic. The physical examination was negative except for pallor. Growth and development were normal. Laboratory data were as follows: volume of packed red cells (VPRC), 36.5 ml./100 ml.; mean corpuscular volume (MCV), 98 μm²; mean corpuscular hemoglobin (MCH) 31 μg; mean corpuscular hemoglobin concentration (MCHC), 32 per cent; leukocyte count (WBC) 11,250/mm.³; leukocyte differential: 3 per cent metamyelocytes, 59 per cent segmented neutrophils, 30 per cent lymphocytes, 6 per cent monocytes, 1 per cent eosinophils, and 1 per cent basophils; platelets 569,000/mm.³, reticulocytes 2.7 per cent. Two morphologically distinct populations of erythrocytes were seen on the blood smear: one which appeared relatively normal, and another which was extremely microcytic and hypochromic. The bone marrow aspirate was cellular with a myeloid:erythroid cell ratio of 1.3:1. About half of the normoblasts were characterized by ragged, scanty, basophilic cytoplasm and the presence of large blue inclusions resembling Pappenheimer bodies. Plasma iron values on three separate occasions were 161, 210, and
SIDEROACHRESTIC ANEMIA

Table 1.—Laboratory Data in Family Members (June, 1966)

<table>
<thead>
<tr>
<th></th>
<th>VPRC ml./100 ml.</th>
<th>MCV μl.</th>
<th>MCHC %</th>
<th>Retic. %</th>
<th>Plasma Fe/μg./100 ml.</th>
<th>Plasma Cu μg./100 ml.</th>
<th>Microcytes %</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. N. (propositus)</td>
<td>32</td>
<td>92</td>
<td>32</td>
<td>2.2</td>
<td>210/194</td>
<td>104</td>
<td>32</td>
</tr>
<tr>
<td>Vi. N. (father)</td>
<td>49</td>
<td>105</td>
<td>32</td>
<td>2.1</td>
<td>85/262</td>
<td>121</td>
<td>0</td>
</tr>
<tr>
<td>J. N. (mother)</td>
<td>43</td>
<td>107</td>
<td>32</td>
<td>2.2</td>
<td>271/313</td>
<td>138</td>
<td>6</td>
</tr>
<tr>
<td>S. N. (sister)</td>
<td>44</td>
<td>92</td>
<td>33</td>
<td>1.2</td>
<td>153/336</td>
<td>110</td>
<td>0</td>
</tr>
<tr>
<td>R. N. (sister)</td>
<td>46</td>
<td>96</td>
<td>33</td>
<td>1.2</td>
<td>101/</td>
<td>112</td>
<td>0</td>
</tr>
<tr>
<td>Ja. N. (sister)</td>
<td>42</td>
<td>87</td>
<td>32</td>
<td>1.4</td>
<td>144/313</td>
<td>127</td>
<td>13</td>
</tr>
</tbody>
</table>

VPRC, volume of packed red cells; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; Retic., reticulocytes; Fe, Iron; T.I.B.C., total iron-binding capacity; Cu, copper.

266 μg./100 ml., and the total iron-binding capacity was 266 μg./100 ml. The bilirubin was 0.2/0.4 mg./100 ml. (direct/total), and the fecal urobilinogen 40 Ehrlich units/100 Gm. A six day therapeutic trial of pyridoxine, 20 mg. per day intramuscularly, resulted in no significant change in the reticulocyte count or in the hemoglobin concentration.

The patient was not seen again by us until March 1964, when she was 14 years old. She had been asymptomatic in the interval and had continued to grow and develop normally. The physical examination was again normal except for slight pallor. The VPRC was 30 ml./100 ml., WBC 8,400/mm.³ platelets 522,000/mm.³, reticulocytes 2.1 per cent, MCV 102 μl.³, and MCHC 35 per cent. Again two erythrocyte populations were seen in the blood smear. The plasma iron was 191 μg./100 ml. and the total iron-binding capacity was 214 μg./100 ml. Values for red cell glutathione, glucose-6-phosphate dehydrogenase activity, and autohemolysis were within normal limits. Direct and indirect Coombs’ tests were negative. Hemoglobin electrophoresis disclosed only hemoglobin A except for 2.6 per cent hemoglobin A₂. Fetal hemoglobin as determined by alkaline denaturation was 0.5 per cent.

Two years later, in June 1966, the physical examination was not significantly changed. Hematologic laboratory data are recorded in Table 1. Again, erythrocyte dimorphism was observed (see Fig. 3A). The bone marrow was as described in 1956. Prussian blue staining disclosed that most of the normoblasts with scanty cytoplasm were “ringed” sideroblasts. Reticuloendothelial iron was normal. Urinary iron excretion after the intramuscular administration of 500 mg. of desferrioxamine was 0.9 mg./24 hr. (normal: 0.3 to 1.0 mg./24 hr.). Ferrokinetic studies were performed with ⁵⁹iron; the plasma iron turnover rate was 149 mg./day (normal: 20 to 42 mg./day) and the red cell iron⁵⁹ utilization 30 per cent (normal: 70 to 90 per cent). The patient received 100 mg. of pyridoxine per day by mouth for three months with no change in the VPRC.

FAMILY STUDIES

The immediate family of the proband (Fig. 1) consisted of the father, age 43; the mother, age 41; and three sisters, ages 19, 14, and 10. The mother had
Fig. 1.—“N” family pedigree indicating the incidence of the erythrocyte defect and the Xg^a blood group antigen.

Table 2.—Blood Group Phenotypes in the “N” Family

<table>
<thead>
<tr>
<th>Subject</th>
<th>Rh</th>
<th>MNS</th>
<th>P</th>
<th>Kell</th>
<th>Lewis</th>
<th>Duffy</th>
<th>Whole Blood</th>
<th>Isolated Microcytes</th>
<th>Isolated Normal Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. N. (propositus)</td>
<td>DCcee</td>
<td>MMSs</td>
<td>+</td>
<td>kk</td>
<td>Le^a-b^+</td>
<td>Fy^a+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VI. N. (father)</td>
<td>DcEe</td>
<td>MNSs</td>
<td>+</td>
<td>kk</td>
<td>Le^a-b^+</td>
<td>Fy^a-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>J. N. (mother)</td>
<td>DCcEe</td>
<td>MMSS</td>
<td>+</td>
<td>kk</td>
<td>Le^b+b^+</td>
<td>Fy^a+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S. N. (sister)</td>
<td>DCcEe</td>
<td>MMSS</td>
<td>+</td>
<td>kk</td>
<td>Le^b+b^+</td>
<td>Fy^a+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>R. N. (sister)</td>
<td>DCcEe</td>
<td>MNSS</td>
<td>+</td>
<td>kk</td>
<td>Le^b+b^+</td>
<td>Fy^a+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ja. N. (sister)</td>
<td>DCcEe</td>
<td>MNSS</td>
<td>+</td>
<td>kk</td>
<td>Le^b+b^+</td>
<td>Fy^a+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

experienced two spontaneous abortions, both at about two to three months of gestation. In both instances, fetal sex was unknown.

All family members were in good health, and the physical examinations were within normal limits except for the presence of a barely palpable spleen in Ja. N., the 10 year old sibling.

Hematologic data in family members are recorded in Table 2. A small population of hypochromic erythrocytes was seen on blood smears from the mother (J.N.) and one sister (Ja.N.). In both cases, the proportion of microcytic cells was small by comparison with that of the proband, and neither subject was anemic. The mother’s serum iron concentration was found to be above normal.

In addition to the immediate family, blood smears were examined from
twenty relatives of the mother (J.N.)* and eighteen relatives of the father (V.N.). All were found to be normal. Of particular genetic significance were the studies in the maternal grandmother of the proband. In this lady, blood smears, serum iron, and VPRC were normal on two examinations. The maternal grandfather was deceased.

**SPECIAL STUDIES**

Erythrocytes were separated according to density by centrifuging in layered gum acacia solutions. All erythrocytes from normal subjects were found at the bottom of the centrifuge tube after this procedure, indicating that their specific gravity was greater than that of the lower gum acacia layer (1.060).

With blood from the three subjects in whom two erythrocyte populations had been found, a visible fraction of the erythrocytes was found at the interface between the lower and upper (Sp. Gr. 1.050) gum acacia layers, indicating that these cells had a specific gravity between 1.050 and 1.060 (Fig. 2). The fraction of erythrocytes at the interface appeared greatest when the blood of the proband was used. Stained smears of the two fractions disclosed that the microcytic population was concentrated in the upper (light) fraction (Fig. 3C) and that the lower (heavy) fraction consisted of normal cells (Fig. 3B).

**Serologic Studies of Erythrocyte Antigens**

The major blood group of all family members was type O. Other common blood group phenotypes are recorded in Table 2 along with that of the X-
linked blood group, Xg\textsuperscript{a}. These studies were consistent with the purported paternity. Since the father’s red cells were Xg\textsuperscript{a}-negative and those of all four daughters were Xg\textsuperscript{a}-positive, the daughters were presumed to be heterozygous with respect to this trait.

Erythrocytes from the three affected subjects were separated by the gum acacia technic and the isolated populations tested for the Xg\textsuperscript{a} antigen. When cells from the proband (V.N.) and her sister (J.N.) were studied, the normal erythrocytes were found to be Xg\textsuperscript{a}-negative and the microcytic erythrocytes were Xg\textsuperscript{a}-positive. In the mother (J.N.), both populations were Xg\textsuperscript{a}-positive. These studies were repeated three times with erythrocytes from V.N. and J.N. with identical results.

Studies of the chromosomes of peripheral lymphocytes disclosed normal karyotypes in all three affected individuals.

Fig. 3.—Photomicrographs of smears made from the whole blood of the propositus (A) compared with the isolated normal (B) and abnormal (C) erythrocyte populations.
Red Cell Porphyrins and Porphyrin Synthesis

Free erythrocyte porphyrins and the synthesis of porphyrins and porphobilinogen from delta-aminolevulinic acid were measured in the three affected individuals (Table 3). Free erythrocyte protoporphyrin was in the low normal range. No evidence for a defective porphyrin biosynthetic enzyme was found when the mixed red cell populations were used. Since normal enzyme levels in the normal population could have obscured a biosynthetic defect in the abnormal population, additional studies were performed on isolated cell populations from the propositus. Both normal and abnormal cells converted delta-aminolevulinic acid to subsequent metabolites at a normal or increased rate.

Free erythrocyte protoporphyrin content was measured in isolated red cell populations from the propositus on three occasions (Table 4). Each time, the porphyrin content of the microcytic population was less than that of the normal population. The porphyrin content was reported in the standard fashion, that is in terms of volume of red cells. This method of reporting tends to minimize the differences observed between the two cell lines. If, for example, the determinations were reported in terms of the number of red cells, the difference would be greater because of the microcytosis of the abnormal population. An apparent increase in the protoporphyrin content of the abnormal cells was observed after pyridoxine therapy, and the observed difference between the normal and abnormal cells was smallest at that time.
DISCUSSION

The proband was found to have moderately severe, chronic anemia, possibly lifelong in duration. The anemia was associated with the presence of two morphologic types of erythrocytes: a relatively normal population and an extremely microcytic and hypochromic population. Two populations of normoblasts could also be seen in the bone marrow. The abnormal erythrocyte precursors were characterized by scanty, basophilic cytoplasm and by the presence of iron-containing inclusions surrounding the nucleus.

The hereditary transmission of the trait was established by documenting similar morphologic changes in the mother and in one of the three siblings. In these two affected relatives, the abnormal population was smaller than in the proband. Possibly for this reason, no anemia was present.

The morphologic picture was similar to that seen in female carriers of hereditary X-linked anemia. However, genetic data alone could not establish X-linkage in this family, since there were no males at risk. It is tempting to speculate that a defect as severe as the one seen in this family would be lethal in the hemizygous (male) state. There were, in fact, two spontaneous abortions reported by the heterozygous mother, but the fetal sex was unknown. This speculation is at odds with the failure to find a similar defect in the maternal grandmother; to maintain it, it would be necessary to propose that the defect arose by mutation in the germ plasm of that individual.

The case for X-linkage of the erythrocyte abnormalities rests upon studies of the Xg* reactivity of the isolated erythrocyte populations. Since the father was Xg*-negative and the two affected daughters were Xg*-positive, it follows that the daughters were heterozygous for this antigen. The red cell abnormality was inherited through the maternal line, and if the Lyon hypothesis can be applied to both the erythrocyte defect and the Xg* antigen, then the normal cells originated from a clone in which the paternal X chromosome was active. In both children, the normal cells were, indeed, Xg*-negative, while the abnormal cells were Xg*-positive. Both the abnormal and normal populations were Xg*-positive in the mother, from which we presume that she is homozygous with respect to the gene controlling the Xg* antigen, a presumption supported by the Xg* type of the unaffected siblings. A diagram of the proposed hereditary mechanism operating in this family appears in Figure 4.

The above data appear to establish that the erythrocyte defect is X-linked and that the phenomenon of X-inactivation applies both to the red cell disorder and to the Xg* antigen. Random variation in the degree of X-inactivation might explain the variation in the size of the abnormal population in the three affected individuals.

Beutler and co-workers were the first to present evidence of red cell mosaicism occurring as a result of X-inactivation in a human disorder, namely, glucose-6-phosphate dehydrogenase (G-6-PD) deficiency. Subsequent studies provided additional evidence that females heterozygous for G-6-PD deficiency have two populations of erythrocytes, one with a normal enzyme content and one with a markedly decreased enzyme content.

Gorman, et al. studied the Xg* antigen in erythrocytes from females hetero-
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Fig. 4.—Diagram illustrating the proposed mechanism of heredity in the “N” family. Each X chromosome carries a gene controlling the Xga blood group and another (E) controlling the erythrocyte changes. The paternal chromosome (X") gives rise to Xga negative, morphologically normal erythrocytes. One maternal chromosome (X12) gives rise to Xga-positive, microcytic erythrocytes.

zygous for G-6-PD deficiency. These investigators tried to separate the two erythrocyte populations by means of a differential agglutination technic with Xga antiserum. Although their technic was effective with artificial mixtures of Xga-positive and Xga-negative cells from male hemizygotes, no separation occurred with cells from females who were heterozygous for both the enzyme defect and the Xga trait. They concluded that X-irractivation did not apply to the Xga locus. It is possible, but unlikely, that the difference between their results and ours indicates that Xga gene inactivation occurs in some clinical conditions, but not in others. More likely, the explanation lies in the methods used. In our patients, it was possible to separate the erythrocyte populations prior to testing with Xga antiserum. In this way, we were able to avoid possible problems arising from the presence of nonspecific antibodies in the antiserum. In this connection, a brief report by Reed and co-workers using fluorescent antibody technics suggested that X-inactivation did not apply to the Xga locus, since more than 99 per cent of cells of female hemizygotes bound the fluorescent label. However, this group later withdrew the above conclusion because the fluorescent antibody label was found to be nonspecific.

The morphologic characteristics of the abnormal population, including the hypochromia, the microcytosis, and the “ringed” sideroblasts in the bone marrow, suggested a disorder of hemoglobin synthesis. This suggestion was supported by high values for serum iron in two of the three affected subjects
and by the increased plasma iron turnover rate and decreased red cell iron utilization in the proband. These observations fit Heilmeyer's criteria for the diagnosis of sideroachrestic anemia.\textsuperscript{23}

It is difficult to exclude thalassemia minor with certainty. However, the family was not of Mediterranean ancestry, and the proportions of fetal and A\textsubscript{2} hemoglobin were normal. Moreover, distinctly dimorphic erythrocyte populations are not seen in that disorder and it is not X-linked.

We suggest, therefore, that the defect lies in porphyrin biosynthetic pathways. This suggestion is supported by the finding of reduced free protoporphyrin levels in the abnormal erythrocyte population. Low values for erythrocyte protoporphyrin have been described in pyridoxine-deficient swine\textsuperscript{24} and in certain patients with pyridoxine-responsive anemia.\textsuperscript{25} In both situations, defective heme biosynthesis has been proposed as an explanation for the low porphyrin values.

In our patients, no defect could be demonstrated in any of the steps by which delta-aminolevulinic acid (ALA) is converted to protoporphyrin. In fact, these conversions occurred at high normal or slightly increased rates (Table 3). By exclusion, therefore, we suggest that the defect lies either in the step by which ALA is synthesized or in some step prior to ALA synthesis. Such a defect has been implicated in other patients with X-linked anemias, primarily because some of these patients respond to pyridoxine,\textsuperscript{26} a derivative of which is known to be a cofactor for ALA synthesis. Pyridoxine therapy in our patient did not result in hematologic improvement, although there was a small and possibly insignificant increase in the protoporphyrin content of the abnormal cells. The ability to respond to pyridoxine may relate to the severity of the defect. For example, if the enzyme ALA synthetase is altered in such a way as to mildly distort the pyridoxal-phosphate-binding site, supranormal levels of the vitamin might restore activity.\textsuperscript{27} In the case of a more severe alteration of the co-enzyme binding site or a defect altering another part of the enzyme, pyridoxine might be ineffective.

**Summary**

1. Two morphologically distinct populations of erythrocytes were found in a mother and in two of her daughters, one of whom (the proband) was anemic. One erythrocyte population was morphologically normal; the other was hypochromic and microcytic.

2. The X-linked blood group antigen, X\textsuperscript{a}, was present in erythrocytes from the mother and the two daughters, but not in erythrocytes from the father. The daughters were, therefore, heterozygous for the gene controlling this antigen.

3. Separation of the two populations of erythrocytes was accomplished by centrifugation in layered gum acacia solutions of different specific gravity.

4. The microcytic cells from the three affected individuals were X\textsuperscript{a}-positive. Isolated normal cells were X\textsuperscript{a}-positive in the mother, but negative in both daughters.

These data suggest that the erythrocyte defect is X-linked and that the
phenomenon of X-inactivation applies to genes controlling both the morphologic defect and the Xg\(^a\) antigen.

5. The free protoporphyrin content of the isolated microcytes was lower than that of the normal cells. The capacity of the microcytes to convert delta-aminolevulinic acid to protoporphyrin was unimpaired.

On these bases it is suggested that the hereditary defect lies either at or before the step in which delta-aminolevulinic acid is synthesized.

**SUMMARIO IN INTERLINGUA**


2. Le antigeno (ligate a X) de gruppo sanguinee, Xg\(^a\), esseva presente in erythrocytos ab le matre e le duo filias. Illo esseva absente ab le erythrocytos del patre. Per consequente, le filias esseva heterozygotic pro le gen que governa le antigeno Xg\(^a\).

3. Le separation del duo populationes de erythrocytos esseva complete per centrifugation in stratificate solutiones de gummi acacia de differente gravitates specific.

4. Le cellulas microcytic ab le tres afferite subjectos esseva Xg\(^a\)-positive. Isolate cellulas normal ab le matre esseva etiam Xg\(^a\)-positive. Isto non esseva le caso quanto a isolate cellulas normal ab le filias.

Iste datos suggestiona que le defecto erythrocytic es ligate a X e que le phenomeno del inactivation de X se applica a genes que regula tanto le defecto morphologic como etiam le antigeno Xg\(^a\).

5. Le contento de protoporphyrina libere in isolate microcytos esseva plus basse que illo in cellulas normal. Le capacitate del microcytos a converter acido delta-aminolevulinic ad in protoporphyrina esseva intacte.

A base del supra-listate constatationes, il es suggestione que le defecto precede le passo del synthese de acido delta-aminolevulinic o coincide con illo.

**ACKNOWLEDGMENTS**

We are indebted to Dr. Joseph D. Mann, Butterworth Hospital, Grand Rapids, Michigan for performing the initial tests for the Xg\(^a\) antigen, to Dr. Fenimore T. Johnson of Upjohn Laboratories for supplying us with heparin, and to Miss Jacqueline Thomas for technical assistance.

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