Primary Acquired Red Cell Hypoplasia Associated With a Clonal Chromosomal Abnormality and Disturbed Erythroid Proliferation

By Peter H. Fitzgerald and Jack W. Hamer

A 43-yr-old male patient with primary acquired red cell hypoplasia was studied over a course of 5 yr. Repeated bone marrow examinations showed marrow replacement by an abnormal cell clone characterized by deletion of most of the long arm of a B group chromosome (Bq−). Chromosomes of blood lymphocytes and skin fibroblasts were normal. ⁵⁵Fe labeling of bone marrow metaphases demonstrated the abnormal chromosome in developing erythroid cells, and it was considered to be present in the common erythroid-myeloid precursor stem cell and its derivatives. Studies of bone marrow cell proliferation using ³H-thymidine autoradiography combined with DNA cytochemistry demonstrated a specific breakdown of erythroid differentiation at the stage of the early polychromatophilic normoblast. It is suggested that red cell hypoplasia was a function of the abnormal clone that replaced normal erythropoietic stem cells of the bone marrow, but proved to be defective in the metabolism required for red cell production. Such replacement of normal bone marrow tissue, presumably associated with the somatically acquired cytogenetic change, is regarded as a neoplastic though nonmalignant development. The patient died from complications of influenza without evidence of tumor or leukemia.

Pure red cell hypoplasia is an uncommon disorder characterized by absent or decreased erythropoiesis and severe anemia with reticulocytopenia, coupled with normal granulocytic and platelet production without evidence of extra medullary erythropoiesis.¹ It has different etiologies, as is shown by the useful classification of DiGiacomo, Furst, and Nixon.² The present paper describes a case of the rare primary acquired type of red cell hypoplasia, studied over a period of 5 yr, during which the bone marrow showed a constant clonal chromosomal abnormality, and disturbed erythroid proliferation.

Case Report

J.H.D., a 43-yr-old male, presented in 1965 with a 6-wk history of weakness, pallor, and tiredness. Physical examination was noncontributory apart from clinical signs of...
anemia; in particular, there was no evidence of hepatic or splenic enlargement or lymphadenopathy.

Past history was uninformative except that a blood count in 1959 had shown a normal hemoglobin. His occupation was that of farmer. He had used an anthelmintic 2-(4-thiazole) benzimidazole on sheep by throat insufflation over the previous 2 yr, but contact with Dr. E. G. McQueen at the New Zealand National Poisons Centre, Dunedin, did not disclose information that the use of this substance had been associated with hematological abnormalities. The patient had no other drug history.

Investigations showed a normochromic normocytic anemia of 5.5 g/100 ml, packed cell volume 17%, reticulocytes 1%, platelets 680,000, and a total white count of 6000, 66% polymorphs, 33% lymphocytes, 1% monocytes.

Sternal marrow showed decreased red cell activity, possible mild myeloid depression with a moderate lymphocytosis of 44%. The myeloid erythroid ratio was 10:1. Megakaryocytes were normal. Iron was present and no ringed sideroblasts were seen. Erythroid precursors were PAS-negative.

Chest and skeletal X rays, intravenous pyelogram, liver function tests, Coombs’ test, serum proteins, electrophoresis, urea, and sugar, were all normal. Creatinine clearance was 153 ml/min. Serum vitamin B₁₂ was 760 µg, and serum folate 8.3 µg. A Schilling test showed normal excretion of vitamin B₁₂. T ⁴²Fe plasma iron clearance was 4.16 hr (normal 1.5–2 hr), with the serum iron 220 µg/100 ml, and TIBC 400 µg/100 ml. ⁴²Fe-red cell utilization at 5 days was 25% (normal > 80%). Surface counting of iron uptake showed the marrow to have 55–75%, the spleen 150% and the liver 250% of the blood activity. T ⁵¹Cr red cell survival was 23 days with no fecal loss of chromium. Fecal urobilinogen excretion was 373–416 mg/day.

He was transfused, and over the next 5 yr had a total of 243 units of packed cells. Numerous blood examinations always disclosed a normal platelet count, white count, and differential, with no immature forms. Reticulocytes were always less than 1%.

Nine bone marrows were studied over the period of 5 yr and they basically continued to show the same picture of reduced erythropoiesis, although later marrows showed a decrease in the original myeloid:erythroid (M:E) ratio ranging from 2:1 to 5:1, with normal granulocytic and megakaryocytic activity. The lymphocyte count was not as prominent in the later marrows, varying from 20 to 24%.

Unsuccessful therapeutic endeavors included courses of vitamin B₁₂, folic acid, pyridoxine, riboflavin, cobaltous chloride 100 mg daily for a period of 2 mo, prednisone averaging 20 mg a day over 2 yr, and oxymethalone 100 mg daily for 18 mo. All of these measures failed to alter the transfusion requirements.

Splenectomy was undertaken in March 1970, because of increasing transfusion requirements and a T ⁵¹Cr red cell survival of 20 days. The 380-g spleen was histologically normal, apart from marked hemosiderosis. Transfusion requirements then returned to their previous level.

Death occurred from cardiac complications of influenzal pneumonia in July 1970. Necropsy showed hemosiderosis involving liver, pancreas, bone marrow, myocardium, lymph nodes, adrenals, and to a lesser extent the skin. There was no lymphadenopathy or evidence of an anterior mediastinal tumor.

Materials and Methods

Chromosomes

Chromosomes were examined in five sternal bone marrow aspirates taken during the period 1965–1970. Marrow specimens were placed directly into colchicine solution, (0.1 µg/ml) for 1 hr, treated with hypotonic KCl solution (0.075 M) for 15 min and fixed in 1:2 acetic-methanol. The fixative was replaced twice during the following 30 min. Blood leukocytes were cultured with phytohemagglutinin (PHA) and prepared according to the general method of Hungerford. Skin biopsies from the right ankle and left arm were cultured as described by Harnden and Brunton. Bone marrow, blood, and skin cells were spread on glass slides by a “flame” method and stained with Giemsa.
Isotopic Iron Labeling of Metaphase Cells

Newly aspirated bone marrow was added to supplemented culture medium 199 plus AB serum and colchicine, as described previously.65 55FeCl3 (Radiochemical Centre, Amersham) was added to give a final concentration of 20 μCi/ml, and the cultures were incubated at 37°C for 12 hr. The cells were then washed in Hanks saline, incubated in hypotonic KCl for 10 min at 37°C, and fixed in 1:3:6 formaldehyde-acetic-methanol for 10 min. The cells were resuspended in fresh fixative and spread by a flame method. Autoradiographs were prepared and processed as described below, except for 21 day’s exposure at 4°C. Giemsa-stained, 55Fe-labeled metaphase cells were photographed, and the silver grains were then bleached from the slides to enable chromosome identification.

A metaphase cell was considered to be labeled with isotopic iron if the area of its spread contained 2 or more times the number of grains in equivalent areas of neighboring background.

Quantitative Cytometry and Autoradiography

Newly aspirated bone marrow cells were suspended in Hanks’ solution containing 3H-thymidine (specific activity 5 Ci/mM, Radiochemical Centre, Amersham) at a concentration of 2 μCi/ml and incubated at 37°C for 1 hr. The medium was removed and the cells were washed and resuspended in AB serum and smeared on glass slides. The air-dried smears were fixed in methanol and stained with Leishman-Giemsa. Bone marrow cells were identified as basophilic normoblasts, early polychromatic normoblasts, late polychromatic normoblasts, myelocytes, and lymphocytes on photographic maps of the smears. The cells were then destained by immersion in 5% trichloracetic acid for 30 sec and absolute methanol for 15 min. The cells were hydrolyzed in normal HCl for 10 min at 60°C, and stained by the Feulgen procedure. Cells were identified from the photographic maps, and the light absorption of their Feulgen-stained nuclei measured by a Barr and Stroud CN2 integrating microdensitometer. The density of Feulgen stain was taken to be a measure of cell DNA content. Slides were then covered with Kodak AR 10 stripping film, exposed for 3 days, and developed in Kodak D19 developer. The cells were reidentified from the photographic maps and 3H labeling scored. This is a modified form of the method described by Wickramasinghe et al.7

RESULTS

Cytogenetics

Cytogenetic studies of bone marrow cells were remarkable for three features: deletion of part of a B chromosome, the presence of degenerate mitotic cells, and the presence of a C chromosome with an elongated secondary constriction.

Nearly all of the bone marrow metaphases contained only 3B group chromosomes and an additional metacentric element which was indistinguishable from the No. 16 pair. These abnormalities are consistent with deletion and loss of part of a B chromosome (Bq-), as is indicated in the karyotype of Fig. 1. Only 5 out of 109 bone marrow cells that were analyzed in detail did not have the deletion (Table 1). This deletion of a B chromosome clearly characterized an abnormal cell line, which constituted the majority of the bone marrow cells during the 5-yr period. The abnormal B chromosome was not present in blood lymphocytes cultured with PHA, nor in cultured skin fibroblasts (Table 1), which indicates that the chromosome abnormality was not constitutional, but a clonal development involving the hemopoietic cells of the bone marrow. Bone marrow, blood, and skin cells with less than 46 chromo-
Fig. 1.—Karyotype of bone marrow metaphase showing the deleted B chromosome (Bq−) (black arrow) and the elongated secondary constriction of a C chromosome (white arrow).

Somes showed a random absence of chromosomes, which presumably resulted from loss during preparation.

Some mitoses in bone marrow cells had a degenerate appearance with small, eroded chromosomes (Fig. 2). The frequency of these mitoses increased from 1 to 53% during the 5-yr period of examination, an increase which corresponded to normalization of the M:E ratio over this period (Table 2). These

Table 1.—Chromosome Studies of the Patient With Primary Acquired Red Cell Hypoplasia

<table>
<thead>
<tr>
<th>Date</th>
<th>Tissue</th>
<th>Chromosome Numbers</th>
<th>Bq-*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/28/65</td>
<td>Bone marrow 1</td>
<td>44 29 29 20 1 8</td>
<td></td>
</tr>
<tr>
<td>6/22/66</td>
<td>Bone marrow 2</td>
<td>45 26 29 25 1 3</td>
<td></td>
</tr>
<tr>
<td>4/1/69</td>
<td>Bone marrow 3</td>
<td>46 21 24 18 6</td>
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</tr>
<tr>
<td>12/5/69</td>
<td>Bone marrow 4</td>
<td>1 - 5 22 27 26 5 1</td>
<td></td>
</tr>
<tr>
<td>7/6/70</td>
<td>Bone marrow 5</td>
<td>1 - 1 25 25 15 3 8</td>
<td></td>
</tr>
<tr>
<td>5/6/69</td>
<td>Blood 1</td>
<td>44 27 30 1 30</td>
<td></td>
</tr>
<tr>
<td>7/23/70</td>
<td>Blood 2</td>
<td>45 33 33 33 -</td>
<td></td>
</tr>
<tr>
<td>12/5/69</td>
<td>Skin (right ankle)</td>
<td>44 22 33 33 -</td>
<td></td>
</tr>
<tr>
<td>7/6/70</td>
<td>Skin (left arm)</td>
<td>46 20 28 28 -</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers of cells in which deletion (Bq−) was present (+), absent (−), or uncertain (?).
degenerate mitoses were not present in the preparations of blood and skin cells.

One chromosome of the C group had a prominent, elongated secondary constriction which in many cells gave it a length equal to that of the B chromosomes (Fig. 1). The constriction was characteristically light-stained and appeared to be an abnormal elongation of the secondary constriction usually present in one C group pair, often designated as C9. The constriction was visible in most cells, and was abnormally elongated in about 25% of bone marrow and skin cells. Marked elongation occurred in nearly all PHA-cultured lymphocytes from the blood. The presence of the abnormality in cells from all tissues examined shows it to be a constitutional one. A study of the patient’s family suggested a dominant pattern of inheritance, the elongated constriction being found in cultured blood cells from the patient’s mother, all of his three sibs, and in two of his three children. This degree of elongation of the C9 secondary constriction is an infrequent occurrence in normal persons.

**Fe Labeling of Bone Marrow Metaphase Cells**

Eighty three metaphase cells from bone marrow aspirate No. 5 were scored

<table>
<thead>
<tr>
<th>Mitotic Cells</th>
<th>Normal</th>
<th>Polyplaid</th>
<th>Degenerate</th>
<th>Total Cells</th>
<th>M:E Ratio</th>
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<tr>
<td>Bone marrow 1</td>
<td>98</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>11:1</td>
</tr>
<tr>
<td>Bone marrow 2</td>
<td>91</td>
<td>—</td>
<td>9</td>
<td>100</td>
<td>6:1</td>
</tr>
<tr>
<td>Bone marrow 3</td>
<td>65</td>
<td>—</td>
<td>35</td>
<td>100</td>
<td>3:1</td>
</tr>
<tr>
<td>Bone marrow 4</td>
<td>66</td>
<td>1</td>
<td>33</td>
<td>100</td>
<td>5:1</td>
</tr>
<tr>
<td>Bone marrow 5</td>
<td>45</td>
<td>2</td>
<td>53</td>
<td>100</td>
<td>4:1</td>
</tr>
</tbody>
</table>
as having the Bq− deletion, and 28 of these were labeled with isotopic iron, the remainder being unlabeled. Of 24 cells without the deletion, 8 were ironlabeled and 16 unlabeled. Very close to one third of both Bq− and normal metaphase cells were labeled. An iron-labeled metaphase cell with the Bq− deletion is shown in Fig. 3.

Bone Marrow Proliferation

Cells from bone marrow aspirates 4 and 5 were examined. Results were similar and only those of aspirate 4 are described in detail.

The relative distribution of the bone marrow cell types in different stages of the cell cycle is shown in Table 3. G1 represents cells with a diploid (2c) content of DNA as determined by Feulgen microdensitometry, S represents cells synthesizing DNA as determined by 3H-thymidine autoradiography, and G2 represents cells that have completed DNA synthesis and are in a premitotic stage with a doubled amount (4c) of DNA. Tritium-labeled cells typically show amounts of DNA covering the whole range between 2c and 4c. However,

| Table 3.—Distribution of Bone Marrow Cell Types in Different Stages of the Cell Cycle |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                |                  |                  |                  |                  |                  |
|                                | G1   | S     | G2   | U    | S/G2 | Total Nuclei |
| Basophilic normoblasts         | 31   | 69    | 0    | 0    | 9.0  | 13            |
| Early polychromatic normoblasts | 39   | 37    | 8    | 16   | 4.7  | 38            |
| Late polychromatic normoblasts  | 100  | 0     | 0    | 0    | —    | 129           |
| Myelocytes                      | 80   | 19    | 1    | 0    | 15.0 | 79            |
| Lymphocytes                     | 98   | 0     | 2    | 0    | —    | 56            |
RED CELL HYPOPLASIA

Fig. 4. — Histograms showing the frequency distribution of DNA content (in arbitrary units) of early polychromatic normoblasts from the bone marrow of the patient with primary acquired red cell hypoplasia, and a normal person. (A) Cells labeled with $^3$H-thymidine; (B) Unlabeled cells. Note the unlabeled cells with amounts of DNA between the 2c and 4c amounts in the red cell hypoplasia.

some unlabeled cells (U) may be found with DNA contents lying between the 2c and 4c amounts. These cells are rare in normal marrows but are common in certain pathological marrows. The patient's basophilic normoblasts and myelocytes showed relatively normal distributions of cells in G1, S, and G2. The early polychromatic normoblasts, however, had reduced numbers of G1 cells, increased numbers of G2 cells, and a considerable proportion of unlabeled cells with an intermediate DNA content (U cells). Late polychromatic normoblasts and lymphocytes were largely nonproliferating and in the G1 phase. As Wickramasinghe et al. have demonstrated, the S/G2 ratio gives a reliable parameter of the dividing cell compartment in that it excludes varying numbers of nonproliferating G1 cells, a factor that is particularly important in the case of polychromatic red cell differentiation. The basophilic normoblasts and myelocytes had S/G2 ratios of the order found in normal bone marrow cells, whereas the early polychromatic normoblasts had a notably reduced S/G2 value. The reduced S/G2 ratio and the presence of U-type cells show that proliferation of the dividing polychromatic normoblasts was specifically disturbed. Histograms showing the frequency distribution of the DNA contents of early polychromatic normoblasts from the patient with red cell hypoplasia and from the marrow of a normal person are shown in Fig. 4.

DISCUSSION

This is the first report of a chromosomally marked clonal cell development in acquired red cell hypoplasia, and our patient is remarkable for the presence of this abnormality in the bone marrow for a period of at least 5 yr. Clinically this appears to have been a typical case, and the unsuccessful therapeutic endeavors in his management followed the generally accepted recommendations. It is probable that red cell hypoplasia is a syndrome of diverse etiologies. In a number of published cases, a relationship to thymoma, response to steroids, and more recently the demonstration of antibody to erythroblast nuclei with a response to immunosuppressive drugs has suggested an immunological mechanism in etiology. However some cases of pure red cell hypoplasia could be considered as potentially leukemic conditions
because of the proportion of patients who have subsequently developed leukemia.\textsuperscript{2–8} Chromosomally abnormal cell lines are more commonly found in the bone marrow of patients with leukemia\textsuperscript{13,14} and have also been reported in patients with a wide range of potentially leukemic myeloid disorders.\textsuperscript{15–17} It is our belief that the present patient should be classified within this etiological group, although he did not manifest evidence of leukemia during the 5-yr course of his disease or at necropsy.

The absence of the deleted chromosome in lymphocytes and skin cells of our patient indicates that it was not a constitutional abnormality, but an acquired one that we can assume occurred originally in a bone marrow stem cell. This cell then proliferated clonally to replace nearly all normal erythropoietic bone marrow cells. Clonal development of the chromosomally abnormal cell line would appear to have preceded or coincided with the manifestation of the red cell disorder. That this association is of etiological significance is supported by our demonstration that the erythroid cell series carried the chromosomal deletion.

One third of the bone marrow cells were labeled with isotopic iron, and can be designated as erythroid precursors. This is in general agreement with the proportion of erythroid cells indicated by the M:E ratio of the same marrow aspirate (No. 5). We can assume that the cell population that did not label with iron included myeloid and other elements. The presence of the Bq—marker in both iron-labeled and unlabeled cells would therefore indicate its presence in a bone marrow precursor cell common to the myeloid and erythroid series. A common erythroid-myeloid precursor cell is consistent with our previous results from isotopic iron labelling of bone marrow cells with the Ph\textsuperscript{1} marker in chronic myeloid leukemia.\textsuperscript{5,6} From this demonstration of the chromosomal marker in the erythroid series it is a reasonable assumption that the ineffective erythropoiesis of our patient was a function of the chromosomally abnormal clone.

Some idea of the mechanism of red cell hypoplasia in this patient is obtained from the disturbed proliferation of the early polychromatic normoblasts. Breakdown of erythroid proliferation and differentiation at this point constitutes an important cause of his erythropoietic insufficiency. However, other factors involving changes within the stem cell department or a decreased rate of differentiation of stem cells are possibly also relevant.\textsuperscript{18} A notable expression of abnormal marrow cell function was the proportion of degenerate mitotic cells, which increased remarkably during the course of the disease, apparently in parallel with an increase in the proportion of erythroid elements, as shown by the marrow M:E ratios.

The early polychromatic normoblast appears to be a particularly vulnerable phase of erythropoiesis, because disturbed proliferation of these cells has been reported in a number of red cell disorders, namely, megaloblastic anemia,\textsuperscript{7,19} sideroblastic anemia,\textsuperscript{20} refractory normoblastic anemia,\textsuperscript{21} and thalassemia.\textsuperscript{22,23} This disturbance of erythroid function has been studied most completely in megaloblastic anemia, where it is believed to reflect an extensive biosynthetic failure involving DNA, RNA, and protein synthesis resulting from specific nutritional or environmental causes.\textsuperscript{24} The cell cycle abnormality of
our patient is probably part of a similar metabolic failure, but exhaustive investigation revealed no nutritional or environmental factor, including drugs, that might have caused this abnormality. An apparently identical proliferation defect in thalassemia appears to result from an inherited genetic factor, but acquired red cell hypoplasia of the adult is not a recognized inherited disorder, nor did our patient have a family history. On the other hand, our studies strongly suggest that his hematological abnormality was the result of the abnormal clone that replaced the normal marrow. To do this, the clonal cell line must have had a genetically based selective advantage, which was probably conferred by the cytogenetic change and operative at the level of a common myeloid-erythroid precursor stem cell. However, the newly established cell line, probably again because of its changed genotype, proved defective in the metabolism required for red cell differentiation, although myeloid and megakaryocytic development proceeded normally. As we have seen, cell selection and replacement were determined at the precursor stem cell level, and the outcome need have had no relation to the ultimate functional ability of the dominant cell, even if this adversely determined the survival of the organism as a whole, as is also the case in leukemia and other malignant disease. Such replacement of normal marrow tissue by a cytogenetically marked clone must be regarded as a neoplasm, albeit not a malignant one. We can offer no information regarding cell selection factors involved in marrow cell replacement, but the present patient shows that an active malignant process is not an essential accompaniment. Likewise, the factors that allowed marrow cell replacement did not at the same time release the cells from the normal controls of proliferation and differentiation such as characterizes the malignant cell. Other writers have noted that the presence of a cytogenetically abnormal cell line is not of itself sufficient for the development of leukemia.

We do not think that the abnormal elongation of the secondary constriction of a C group chromosome is causally related to the red cell hypoplasia. This inherited abnormality was present in several family members belonging to three generations, but there was no family history of the disease. Also, we know of several other families that carry this inherited abnormality, and none involves a case of pure red cell hypoplasia. The congenital form of erythroid hypoplasia (Blackfan-Diamond syndrome) has been associated in one instance with an achromatic area affecting a single chromatid of chromosome No. 1. This was possibly a chance association. Similar achromatic areas and chromatid breaks have been reported in Fanconi's anemia, although there must be a strong probability that these are epiphenomena secondary to a more basic metabolic disturbance, as is the case in megaloblastic anemia.

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