Evidence for a Multistep Pathogenesis of a Myelodysplastic Syndrome

By Wendy H. Raskind, Nagendra Tirumali, Robert Jacobson, Jack Singer, and Philip J. Fialkow

Somatic cell genetic approaches utilizing the cellular mosaicism present in women heterozygous for glucose-6-phosphate dehydrogenase (G6PD) have provided information relevant to the pathogenesis of some neoplastic disorders. With these techniques, we studied a 61-year-old woman with a myelodysplastic syndrome. GdA/GdA heterozygosity was demonstrated in skin and cultured T lymphocytes, which exhibited both A and B type G6PD. In contrast, erythrocytes, platelets, granulocytes, and marrow nucleated cells displayed almost exclusively G6PD type B. In addition, 21 of 24 Epstein-Barr virus-transformed B lymphoblastoid lines that expressed a single immunoglobulin light chain showed only type B G6PD, suggesting that the stem cells involved by this disease were clonal and could differentiate to B lymphocytes as well as to mature granulocytes, erythrocytes, and platelets. Cultured skin fibroblasts and phytohemagglutinin-stimulated lymphocytes were karyotypically normal, but two independent abnormalities were found in marrow—47,XX,+8 and 48,XX,del(11)(q23). None of 14 type B G6PD lymphoblastoid lines analyzed in detail contained these karyotypic abnormalities, which strongly suggests that a visible chromosomal alteration is not the sole step in the development of this disease. We hypothesize that at least two events are involved in the pathogenesis of this patient’s myelodysplasia: one causing proliferation of a clone of genetically unstable pluripotent stem cells and another inducing chromosomal abnormalities in its descendants.

MATERIALS AND METHODS

Subject

The patient was found to have a refractory anemia when she presented to George-town University Hospital in 1975 at the age of 61 yr. Laboratory evaluation revealed a hematocrit of 34%, mean corpuscular volume (MCV) of 102 cu μ, reticulocyte count 2.1%, white cell count 3,800/cu mm, with a normal differential, serum iron 137 mg/dl, total iron binding capacity 275 mg/dl, and normal serum folate and vitamin B12 levels. In April 1982, her hematocrit had fallen to 18%, but subsequently stabilized at 31%. Morphological evaluations have consistently shown marked peripheral erythrocyte anisopoikilocytosis and bone marrow erythroid hyperplasia with dysplastic and megaloblastic erythropoiesis and fewer than 5% blasts. The periodic acid-Schiff (PAS) stain has not shown block-positive erythrocyte precursors. Iron stores have been markedly increased, with ringed sideroblasts comprising 5%–15% of the nucleated red cell population. This patient’s course has been presented in abstract form8 and will be described in greater detail elsewhere.

Specimens

Peripheral blood was drawn into sterile tubes containing either EDTA or preservative-free heparin. Marrow was obtained by iliac crest aspiration into heparinized syringes. Samples were air-transported from Washington, D.C. and received in Seattle approximately 24 hr after removal from the patient, who gave informed consent for these procedures.

Cell Culture

All cultures were grown in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 U/ml), and fungizone (0.025 μg/ml) (GIBCO, Grand Island, N.Y.) at 37°C in 5% CO2 and 95% humidity. Peripheral blood leukocytes and marrow were cultured in medium supplemented with 20% fetal calf serum. Phytohemagglutinin (PHA) (Burroughs Wellcome, Research Triangle Park, NC) was added to some blood cultures. Marrow-derived fibroblasts were grown as monolayers with decreasing percentages of fetal calf serum from 30% to 10%.

EBV Transformation

Peripheral blood mononuclear cells were isolated by centrifugation over Isolymph (specific gravity 1.077; Gallard-Schlesinger,
Carle Place, NY) at 800 g for 30 min at 15°C. The interface cells were washed and incubated on ice with AET-treated sheep red blood cells for 1 hr.

The cells were then centrifuged over Percoll (specific gravity 1.077, Pharmacia, Piscataway, NJ) to separate the high density rosettes from the light density, B lymphocyte-enriched nonrosetting mononuclear cells. The latter cells were incubated with equal volumes of filtered 3-day supernatant from the Epstein-Barr virus (EBV) producing line MCUV (generously provided by Dr. Paul Martin) and plated into 96-well microculture trays (10⁶ cells in 0.2 ml/well). Cultures were fed once or twice weekly with medium containing 10% fetal calf serum and expanded by transfer to successively larger wells as required.

G6PD Analyses

Starch gel and cellulose acetate electrophoreses were performed as described. The ratio of A: B enzyme was estimated visually. The sensitivity of this technique allows the detection of a minor enzyme component as small as 5%.

Cytoplasmic Immunoglobulin Determination

Indirect immunofluorescence was performed on cytocentrifuge slides using purified mouse monoclonal anti-human immunoglobulin (Ig) kappa (κ) and lambda (λ) light chains (Becton Dickinson, Mountain View, CA) and fluorescein isothiocyanate-tagged goat anti-mouse IgG (Cappel, West Chester, PA).

Cytogenetics

Marrow and unstimulated peripheral blood cell cultures were harvested at 24 hr; PHA-stimulated blood cultures were grown for 72 hr. All chromosome harvests were initiated by a 2-hr incubation with colchicine (0.02 µg/ml) followed by treatment with hypotonic KCl (0.075 M) and fixation 3 times with 3:1 methanol:glacial acetic acid. G-bands by acetic saline using Giemsa (GAG), G-bands by trypsin using Giemsa (GTG), or R-bands by heat using Giemsa (RHG) banding was done.

RESULTS

Analysis of skin and cultured skin fibroblasts revealed the patient to be heterozygous for G6PD (Gd²/Gd³), with a 1:1 ratio of A:B enzyme. At the time of initiation of cultured B lymphoid cell lines, G6PD studies of blood cell fractions and hematopoietic colonies grown in vitro indicated that granulocytes, platelets, and erythroid cells were clonal (they exhibited almost exclusively type B G6PD). These findings have been published in abstract form¹ and will be described in detail elsewhere.

B Lymphoblastoid Cell Lines

Twenty-seven lines were recovered from 182 plated wells. As has been observed in CML³ and chronic lymphocytic leukemia,¹⁴ the frequency of EBV transformation and the growth rate and duration of survival of the resulting lines were all reduced. Three lines that expressed both G6PD isoenzymes or both light chains were excluded from statistical analysis. Of the 24 lymphoblastoid lines with a single G6PD and light chain phenotype, 21 displayed B type G6PD and 3 displayed A type enzyme. The ratio of lines producing κ and λ light chains was 14:10.

Cytogenetics

On four occasions spanning a 2.5-yr period, marrow was obtained for cytogenetic analysis. The modal karyotype was consistently 46,XX,del(11)(q23). On two occasions, a 47,XX,+8 karyotype was found (Table 1, Figs. 1 and 2). No cell was identified containing both trisomy 8 and 11q- deletion. PHA-stimulated lymphocytes studied on 3 occasions and cultured marrow fibroblasts were karyotypically normal.

The blood sample processed for EBV transformation was obtained on April 14, 1982, when 17 of 18 marrow metaphases revealed the 11q− deletion. None of 14 type B or 2 type A G6PD lines from which at least 5 metaphases could be analyzed in detail contained the clonal karyotypic abnormalities observed in marrow (Table 2).

DISCUSSION

The preleukemic syndromes, including myelodysplasia, are clinically defined disorders that carry an increased risk for ultimate development of acute non-lymphocytic leukemia (ANLL).¹² Study of these preneoplastic states may provide information regarding

<table>
<thead>
<tr>
<th>Date Obtained</th>
<th>Number of Chromosomes</th>
<th>Total Cells Counted</th>
<th>Karyotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;46</td>
<td>46</td>
<td>47</td>
</tr>
<tr>
<td>9-23-80</td>
<td>2</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>3-4-81</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>4-14-82</td>
<td>13</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>1-27-83</td>
<td>9</td>
<td>16</td>
<td>6</td>
</tr>
</tbody>
</table>

For determination of chromosome number, consecutive metaphases were analyzed without selection. Cells with fewer than 46 chromosomes appeared to have resulted from random chromosome loss. Adequately banded metaphases were independently chosen for karyotyping and were not necessarily included in the modal number determination.
the cellular changes that are important in the conversion of a normal cell to a malignant one.

G6PD tests of uncultured blood cells indicated that, at the time of study of B lymphoid cell lines, this patient's myelodysplastic syndrome was clonal and involved stem cells multipotent for erythrocytes, granulocytes, and platelets. Cytogenetic studies on marrow cells revealed the presence of two distinct chromosomal abnormalities. To determine if B lymphocytes also emanated from the stem cell involved by the disease and to investigate the relationship of chromosomal abnormalities to the pathogenesis of the disorder, we established multiple B lymphoblastoid cell lines from this patient's blood cells, as it is not possible currently to determine cell type, karyotype, and G6PD phenotype simultaneously in a single cell. When grown from people without hematologic diseases, these lines become homogeneous with respect to the Ig and G6PD types produced, suggesting overgrowth by progeny of one or a few cells. Specific G6PD and Ig phenotypes do not appear to confer selective advantage. By examining individual lymphoblastoid lines that have arrived at a homogeneous state, it is possible to infer the characteristics of the one or few progenitor cells from which the lines were derived.

At the time of study, after 64–103 days in culture, the great majority of B lymphoid lines analyzed from our patient were monomorphic with respect to G6PD and Ig light chain. The low transformation efficiency observed is consistent with the findings in other patients with hematologic diseases involving B cells.  

---

**Fig. 1.** G-banded karyotype prepared from the 4-14-82 marrow sample.

---

**Table 2. Analyses of Lymphoblastoid Lines**

<table>
<thead>
<tr>
<th>Line showing G6PD type A</th>
<th>Number of Metaphases Examined</th>
<th>Light Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIA3</td>
<td>30</td>
<td>λ</td>
</tr>
<tr>
<td>IIA6</td>
<td>25</td>
<td>κ</td>
</tr>
<tr>
<td>IIA4</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lines showing G6PD type B</th>
<th>Number of Metaphases Examined</th>
<th>Light Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIB3</td>
<td>33</td>
<td>λ</td>
</tr>
<tr>
<td>IIIB4</td>
<td>32</td>
<td>λ</td>
</tr>
<tr>
<td>XIIVA3†</td>
<td>30*</td>
<td>λ</td>
</tr>
<tr>
<td>IA4</td>
<td>30*</td>
<td>κ</td>
</tr>
<tr>
<td>IIIB6</td>
<td>28</td>
<td>κ</td>
</tr>
<tr>
<td>IIIB4</td>
<td>22</td>
<td>λ</td>
</tr>
<tr>
<td>ID6</td>
<td>20</td>
<td>κ</td>
</tr>
<tr>
<td>ID2</td>
<td>14*</td>
<td>λ</td>
</tr>
<tr>
<td>IIB1</td>
<td>11</td>
<td>λ</td>
</tr>
<tr>
<td>IA6</td>
<td>7</td>
<td>κ</td>
</tr>
<tr>
<td>IID3</td>
<td>6*</td>
<td>κ</td>
</tr>
<tr>
<td>IIC3</td>
<td>5</td>
<td>κ</td>
</tr>
<tr>
<td>IID1</td>
<td>0</td>
<td>κ</td>
</tr>
<tr>
<td>IIB6</td>
<td>0</td>
<td>κ</td>
</tr>
<tr>
<td>IIA5</td>
<td>0</td>
<td>λ</td>
</tr>
<tr>
<td>ID5</td>
<td>0</td>
<td>κ</td>
</tr>
<tr>
<td>IIC4</td>
<td>0</td>
<td>κ</td>
</tr>
<tr>
<td>IA2</td>
<td>0</td>
<td>κ</td>
</tr>
<tr>
<td>IIB6</td>
<td>0</td>
<td>κ</td>
</tr>
<tr>
<td>IC1</td>
<td>0</td>
<td>λ</td>
</tr>
</tbody>
</table>

No metaphases with trisomy 8 were observed. In those lines marked by an asterisk (*) a single metaphase that may have exhibited the 11q- chromosome was present.

†XIIVA3 was obtained by subculturing a mixed A/B line and is included only for cytogenetic data. All subcultures of the original line showed only G6PD type B.
Fig. 2. R-banded karyotypes from the marrow sample obtained 1-27-83. (A) The dark bands 11q23-qter missing from one chromosome 11 cannot be located on another chromosome and may have been deleted from the cell. (B) 47,XX,+8. The number 11 chromosomes appear normal.
In previous experiments with EBV-transformed lines, the involvement in CML of a stem cell common to the B lymphoid as well as erythroid and myeloid series was demonstrated by the finding of Ig-synthesizing Ph-positive B lymphoid cells. A similar conclusion was reached from studies of the B-lymphoid cell lines grown from our patient. In a normal Gd\(^B\)/Gd\(^A\) heterozygote with a 1:1 ratio of G6PD B:A in skin, the ratio in blood cells is generally also 1:1. In contrast, the ratio of G6PD type B:A in lymphoblastoid lines from this patient was significantly different from the 1:1 ratio predicted (21B:3A, \(p < 0.01\), \(\chi^2\) with Yates’ correction). It is unlikely that these findings are explained by a selective growth advantage of cells that produce type B G6PD. A more probable explanation is that at least some of the type B lines were derived from the stem cell population responsible for the clonal proliferation of red cells, granulocytes, and platelets, suggesting the involvement in this myelodysplastic anemia of a stem cell pluripotent for myeloid and B lymphoid cells.

B lymphocytes undergo a complex maturation process that results in mature B cells capable of synthesizing a single Ig type. B lymphocytes derived from the pluripotent stem cell clone involved by the chronic phase of CML exhibit Ig diversity. Similarly, of the 21 lymphoblastoid lines producing type B G6PD recovered from this patient, 12 were found to have cytoplasmic \(\kappa\) light chain and 9 had \(\lambda\). In contrast, in CML lymphoblastic crisis and in common-type acute lymphoblastic leukemia, the cells are uniform in their Ig gene rearrangements. These observations indicate that Ig restriction occurs at some intermediate stage in differentiation between the clonal pluripotent stem cells involved by hematologic neoplasms and the malignant lymphoblast.

In marrow cells from this patient we found two karyotypically abnormal clones [47,XX,+8 and 46,XX,del(11)(q23)], ranging from 0% to 26% and 58% to 94% of metaphases, respectively. The deleted bands 11q23-qter could not be located in R- or G-banded metaphases as a translocation. Structural alterations involving the long arm of chromosome 11 have been reported in a variety of hematologic disorders, such as acute monocytic and acute myelomonocytic leukemias. A translocation involving chromosomes 9 and 11 was found in approximately half the marrow metaphases examined during a brief “preleukemic” phase preceding overt acute myelomonocytic leukemia in case 3 reported by Hagemeijer et al. Deletions of 11(q14 or q23-qter) may occur nonrandomly in acquired idiopathic sideroblastic anemia; progression to ANLL has been observed in one of the four reported cases. Neither the acquisition of a third chromosome 8 nor the breakage at band q22-q23 of chromosome 11 in marrow cells has yet led to acute leukemia in our patient, suggesting that the specific karyotypic changes are not sufficient to cause malignancy.

Study of lymphoblastoid cell lines from a patient with CML provided evidence for a population of B cells belonging to the abnormal clone but not containing the Ph chromosome, suggesting that generation of Ph may not be the sole event in the pathogenesis of that patient’s CML. Further, in vitro evidence for chromosome instability in Ph-negative clonal precursor cells was obtained by the demonstration of multiple chromosomal abnormalities in Ph-negative B lymphoid cell lines derived from the abnormal pluripotent stem cell clone. Thus, the data suggested that alterations in karyotype, including the generation of Ph, occur after a step that leads to clonal proliferation of genetically unstable pluripotent stem cells.

As in the case of CML, although the patient reported here had distinctive specific chromosomal abnormalities, their occurrence very probably was not the sole event in the development of her myelodysplasia. The relative frequencies of cells with apparently normal karyotype or with trisomy 8 or 11q- varied in the different marrow samples examined. At the time peripheral blood was obtained for EBV transformation, 1 of 18 marrow metaphases was normal and the remaining 17 exhibited 11q-. However, despite the fact that many of the B-lymphoblastoid cell lines probably arose from cells of the abnormal clone, none of them had +8 or 11q-. One explanation for the failure to detect a chromosomally abnormal clone in these lines is that progenitor cells with either +8 or 11q- were incapable of differentiating to mature B cells. Alternatively, B cells with either of these chromosome changes may have lacked EBV receptors or have been less likely to survive EBV infection. In any event, the existence of these clonal B lymphocytes without visible karyotypic abnormalities provides evidence that clonality of the progenitor cells in this disease occurred prior to the generation of the chromosome changes.

It is very likely that the clonal progenitor cells are genetically unstable. In the patient with CML, evidence for genetic instability of the putative clone of Ph-negative stem cells was provided by the demonstration of multiple chromosome abnormalities in Ph-negative B lymphoid cell lines derived from the abnormal pluripotent stem cell clone. We did not find similar aberrations in lines grown from our patient, but the finding of two independent clonal chromosome abnormalities in marrow cells is evidence for genetic instability of the clone of stem cells from which those cytogenetically aberrant cells arose.

Within the preleukemic syndromes, the cytopenic
anemias carry a high risk for ultimate development of leukemia (at least 25%–50%). In this patient with myelodysplastic anemia, more than one divergence from normal hemopoiesis has already occurred—generation of a clone of pluripotent stem cells and the production of nonrandom chromosome changes in some descendants of these cells. These abnormalities alone or in combination have not been sufficient to cause overt leukemia. Presumably, additional step(s) are required for the loss of proliferative control seen in malignancy. Periodic evaluations of the various hemopoietic compartments in this patient may allow delineation of some of the steps involved in the progression of this myelodysplasia and may provide more information regarding the prognostic significance of 11q alterations.

In summary, we have given evidence that the myelodysplastic syndrome in this patient is a clonal disease arising in a stem cell pluripotent for erythrocytes, granulocytes, platelets, and B lymphocytes. The stem cell is more immature than one with restriction of Ig light chains and has no visible chromosomal changes. However, an important characteristic of this stem cell may be genetic instability, and this attribute may be the one that leads to development of clinically apparent disease.

ACKNOWLEDGMENT

We are grateful to Drs. George Shashaty and Ronald Sacher for referring the patient for study. We thank Gabriel Herner, Armí Salo, Laura Steinmann, and Connie Waddington for their excellent assistance, and Dr. Christine Disteche and Steven Forbes for their cytogenetic advice.

REFERENCES

20. DeWald GW, Morrison-DeLap SJ, Schuchard KA, Spurbeck JL, Pierrr RV: A possible specific chromosome marker for monocytic leukemia: Three more patients with t(9;11)(p22;q24) and another with t(11;17)(q24q21), each with acute monoblastic leukemia. Cancer Genet Cytogenet 8:203, 1983
Evidence for a multistep pathogenesis of a myelodysplastic syndrome

WH Raskind, N Tirumali, R Jacobson, J Singer and PJ Fialkow