Clonal Involvement of Granulocytes and Monocytes, But Not of T and B Lymphocytes and Natural Killer Cells in Patients With Myelodysplasia: Analysis by X-Linked Restriction Fragment Length Polymorphisms and Polymerase Chain Reaction of the Phosphoglycerate Kinase Gene

By Harmen van Kamp, Willem E. Fibbe, Rumo P.M. Jansen, Maarten van der Keur, Esther de Graaff, Roel Willemze, and James E. Landegent

To determine the clonal nature of hematopoiesis and to assess lineage involvement in patients with myelodysplastic syndromes (MDS), we used restriction fragment length polymorphisms of the X-linked genes phosphoglycerate kinase (PGK1) and hypoxanthine phosphoribosyltransferase (HPRT) and the X-linked probe M279. Eleven female MDS patients heterozygous for at least one of these probes were studied: 3 with refractory anemia (RA), 2 with RA with ringed sideroblasts (RARS), 2 with chronic myelomonocytic leukemia (CMML), and 4 with RA with excess of blasts in transformation (RAEB-t). All exhibited clonal hematopoiesis as determined by Southern analysis of DNA prepared from peripheral blood and bone marrow, and the number of peripheral blood monocytes.11,12

MELLODYSPLASTIC syndromes (MDS) are a heterogeneous group of clinically defined disorders characterized by ineffective hematopoiesis involving at least two myeloid lineages. Patients with MDS show peripheral blood cytopenias, and a normocellular or hypercellular bone marrow with signs of a defective maturation.1 A classification into five subgroups has been made by the French-American-British (FAB) Cooperative Study Group based on the number of blast cells in peripheral blood and bone marrow, the presence of ringed sideroblasts in the bone marrow, and the number of peripheral blood monocytes.2 The FAB classification represents a reflection of the progression to leukemia and, therefore, the subtypes correlate with survival. Evidence for the clonal nature of MDS is provided by nonrandom cytogenetic abnormalities that are found in about 50% of patients with primary MDS3,4 and in about 90% of patients with MDS secondary to long-term cytotoxic chemotherapy and/or radiotherapy for other malignant diseases.5,6 The activation of proto-oncogenes may also be used as markers of clonality. In particular, activated ras oncogenes are found in MDS and have been reported to occur in 6% to 40% of cases.7 Further, clonal hematopoiesis has been shown by X-chromosome inactivation studies in female patients using the X-linked enzyme glucose-6-phosphate dehydrogenase (G-6-PD),10,11 and restriction fragment length polymorphisms (RFLPs) of the X-linked genes phosphoglycerate kinase (PGK1) and hypoxanthine phosphoribosyltransferase (HPRT).12,13 Also, the clonal origin of polymorphonuclear cells (PMN) in patients with MDS has been shown by Gilliland et al by polymerase chain reaction (PCR) analysis of the methylation pattern of the PGK1 gene.14 However, studies on the involvement of lymphoid cells in the clonal hematopoiesis of patients with MDS show ambiguous results.

In the present study, we have studied clonality of hematopoietic cells in MDS patients by Southern analysis using X-linked RFLPs in conjunction with their methylation pattern. Analysis of DNA derived from purified suspensions of lymphocytes and/or T cells showed a polyclonal pattern. To study clonality on a limited number of cells, we have used a PCR-based technique to study the methylation pattern of the PGK1 gene.14-16 DNA derived from peripheral blood (PB) and bone marrow (BM) cells, PMN and fluorescence-activated cell sorted (FACS) monocytes, T and B lymphocytes, and natural killer (NK) cells of female MDS patients heterozygous for the PGK1 gene were analyzed. Using this method,
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a clonal pattern was shown in myeloid cells, whereas circulating T and B lymphocytes, and NK cells exhibited a polyclonal pattern.

PATIENTS AND METHODS

Patients. Twenty-one female patients (median age, 65 years; range, 26 to 78 years) with MDS were tested for heterozygosity for the X-linked genes PGK1 and HPRT. Thirteen of these patients were also tested for heterozygosity for the X-linked probe M27P. Ten had refractory anemia (RA), 3 had RA with ringed sideroblasts (RAEB), 3 had chronic myelomonocytic leukemia (CMML), and 5 had RA with excess blasts in transformation (RAEB-t), according to the FAB-criteria. The characteristics of the 11 informative patients are given in Table 1.

Cell separation for Southern analysis. Purified cell suspensions for analysis of clonality by Southern blotting were obtained from three patients (unique patient number [UPN] 01, 04, 05) heterozygous for the PGK1 gene. Leukapheresis of 500 mL blood was performed and purified suspensions of PMN, monocytes, lymphocytes, and (or) T cells were prepared and analyzed for purity as described.7

Cell separation for PCR analysis of the PGK1 gene. Cryopreserved samples of low-density cells derived from PB in three patients (UPN 01, 05, 08) and from BM in two patients (UPN 04, 09) heterozygous for the PGK1 gene, were thawed and incubated with fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies (concentrations according to the manufacturer's instructions) for 30 minutes at 4°C, washed, and resuspended in 0.9% NaCl, 1% bovine serum albumin. Leu-4-FITC and Leu-4-PE (anti-CD45), Leu-3-PE (anti-CD20), Leu-3-FITC (anti-CD45), Leu-3-PE (anti-CD4), Leu-11c-PE (anti-CD16), and Leu-9c-PE (anti-CD56) were obtained from Becton Dickinson (Mountainview, CA).2H4-PE (anti-CD45RA) from Coulter Corporation (Hialeah, FL), and UCHL1-PE (anti-CD2) were obtained from Becton Dickinson, respectively. For isolation of NK cells, the lymphocyte scatter signal was set for lymphocytes and monocytes, respectively. For isolation of NK cells, the lymphocyte scatter gate was extended to include large granular lymphocytes. Cells were subsequently discriminated by two-color flow cytometry and sorted in CD14+CD45+ (monocytes), CD3+ (T lymphocytes), CD20+ (B lymphocytes), and CD16+CD56+CD3-CD14- (NK cells) fractions. CD3+CD45RA+ (naïve T cells) and CD3+CD45RO+ (memory T cells) were also sorted from the cryopreserved samples. Samples of each fraction consisted of 5,000 cells in 0.9% NaCl. The cells were centrifuged at 14,000 rpm, and resuspended in 50 μL distilled water after removal of the supernatant. Subsequently, the samples were boiled and stored at -20°C until processing (see below). DNA derived from either a mouth wash, hair roots, or paraffin-embedded skin biopsy specimen was used as a control to analyse the constitutive methylation pattern in UPN 01, 04, 05, and 08.

Southern analysis. High-molecular weight DNA was prepared from PB and BM cells and from the purified cell suspensions by sodium dodecyl sulfate (SDS)-pronase digestion and subsequent ethanol precipitation. Clonal analysis was performed with probes derived from the X-linked genes PGK1 and HPRT and with the M27B probe as described.7 The probe used for analysis of the PGK1 gene was obtained by PCR amplification using a 5' coding primer (TGT TCC GCA TTC TGC AAG CC) and a 3' antisense primer (GGA AAA TGC GGC TAG AAA CC).8,18 This generated a 333-bp genomic fragment containing the first exon of the gene. For analysis of heterozygosity for the HPRT gene, the plasmid pPB 1.7 was used, containing a 1.7 kb BamHI/PstI fragment from the 5' region of the gene. The methylation of the HPRT gene is complex. There are 9 HpaII sites at the 5' end of the HPRT gene, from which sites 2 to 9 are differentially methylated. In active alleles, sites 2 or 3 are unmethylated, whereas sites 4 through 9 are methylated. There are two types of inactive alleles. In type I alleles, sites 2 and 3 are methylated, and at least one of the sites 4 to 9 is unmethylated. In type II alleles, sites 2 through 9 are all methylated. Two probes for the HPRT gene are used for clonal analysis. The pHPRT-800 probe (containing an 800-bp HpaII/PstI subfragment from pPB1.7 cloned into the AccI/PstI sites of pGEM-4), hybridizing to the gene between sites 2 and 3, can be used for clonal analysis of those women with type I inactive alleles (60% to 100%). The pHPRT-600 probe (containing a 600-bp HpaII

Table 1. Patient Characteristics at the Time of Clonal Analysis

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age (yr)</th>
<th>Diagnosis (FAB)</th>
<th>Time After Diagnosis (mo)</th>
<th>Hemoglobin (mmol/L)</th>
<th>MCV* (fl)</th>
<th>Leukocytes (x10⁹/L)</th>
<th>Neutrophils (x10⁹/L)</th>
<th>Platelets (x10⁹/L)</th>
<th>Cytogenetic Analysis</th>
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<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>RA</td>
<td>104</td>
<td>4.8±</td>
<td>86</td>
<td>1.2</td>
<td>0.5</td>
<td>10</td>
<td>No mitoses</td>
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<tr>
<td>2</td>
<td>77</td>
<td>RA</td>
<td>3</td>
<td>5.2±</td>
<td>111</td>
<td>3.8</td>
<td>1.6</td>
<td>266</td>
<td>46,XX=18 46,XX,−7=2</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>RA</td>
<td>2</td>
<td>5.1±</td>
<td>113res</td>
<td>5.8</td>
<td>2.9</td>
<td>383</td>
<td>46,XX=17 47,XX,+8=3 45,XX,−2,4q+,+10p+</td>
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<tr>
<td>4</td>
<td>55</td>
<td>RARS</td>
<td>102</td>
<td>5.8±</td>
<td>87</td>
<td>6.1</td>
<td>3.9</td>
<td>672</td>
<td>46,XX</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>RARS</td>
<td>72</td>
<td>5.4±</td>
<td>99</td>
<td>5.5</td>
<td>3.7</td>
<td>360</td>
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</tr>
<tr>
<td>6</td>
<td>69</td>
<td>CMML†</td>
<td>11</td>
<td>3.6†</td>
<td>111res</td>
<td>3.7</td>
<td>5.4</td>
<td>37</td>
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<tr>
<td>7</td>
<td>36</td>
<td>CMML</td>
<td>68</td>
<td>6.6†</td>
<td>119</td>
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<td>0.5</td>
<td>84</td>
<td>46,XX</td>
</tr>
<tr>
<td>8</td>
<td>62</td>
<td>RAEB-t</td>
<td>1</td>
<td>5.7†</td>
<td>85</td>
<td>22.5</td>
<td>13.5</td>
<td>66</td>
<td>46,XX</td>
</tr>
<tr>
<td>9</td>
<td>59</td>
<td>RAEB-t</td>
<td>2</td>
<td>5.3†</td>
<td>90</td>
<td>6.4</td>
<td>3.2</td>
<td>18</td>
<td>45,XX=2 46,XX,del(5)(q13;qter),19p+=15 45,XX,−19,del(6)(q13);c(ter)+</td>
</tr>
<tr>
<td>10</td>
<td>73</td>
<td>RAEB-t</td>
<td>2</td>
<td>4.1†</td>
<td>114</td>
<td>6.0</td>
<td>2.4</td>
<td>425</td>
<td>46,XX,−2,−5,del(3)(p14),del(6)(q21);q23, 11q+,+M1,+M2=20</td>
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<tr>
<td>11</td>
<td>78</td>
<td>RAEB-t</td>
<td>29</td>
<td>5.5†</td>
<td>93</td>
<td>1.3</td>
<td>0.1</td>
<td>47</td>
<td>No mitoses</td>
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*Mean corpuscular volume.
†Red blood cell transfusion dependency.
‡Secondary to cyclophosphamide therapy for Wegener's granulomatosis.
Table 2. Clonality of Hematopoiesis in Patients With Myelodysplasia as Assessed by Southern Blotting

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis (FAB)</th>
<th>Probe Used</th>
<th>PB BM</th>
<th>PMN</th>
<th>MO</th>
<th>LY</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RA P</td>
<td>+</td>
<td>+/75</td>
<td>-</td>
<td>/99</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>RA M</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>/95</td>
<td>-</td>
<td>/98</td>
</tr>
<tr>
<td>3</td>
<td>RA M</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>/98</td>
<td>/80</td>
<td>/90</td>
</tr>
<tr>
<td>4</td>
<td>RARS P</td>
<td>+</td>
<td>+/95</td>
<td>-</td>
<td>/99</td>
<td>-</td>
<td>/98</td>
</tr>
<tr>
<td>5</td>
<td>RARS P</td>
<td>+</td>
<td>+/98</td>
<td>+/80</td>
<td>-</td>
<td>-</td>
<td>/90</td>
</tr>
<tr>
<td>6</td>
<td>CMML P,M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>CMML M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>RAEB-t P,M,H</td>
<td>+</td>
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</tr>
<tr>
<td>9</td>
<td>RAEB-t P</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>RAEB-t M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>RAEB-t H</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: MO, monocytes; LY, lymphocytes; T, T cells; C, result of clonal analysis; +, clonal pattern; -, polyclonal pattern; Pu, purity of cell suspensions (%); M, M27P; P, PGK; H, HPRT.

subfragment from pPB1.7 cloned into the AccI site of pGEM-4, hybridizing between sites 1 and 2, can be used for examining the methylation of HpaII sites 2 and 3 in women with type II inactive alleles. The M27P probe (kindly provided by Dr I. Craig from Oxford, UK) is located at Xcen-p11.22 (DXS255) and recognizes a subfragment from pPB1.7 cloned into the vector (Boehringer, Mannheim, Germany) and purified by elution over sephadex G50.

PCR analysis of the PGK1 gene. PCR analysis of the PGK1 gene was performed as described recently with some modifications. Briefly, the fragments to be amplified contain the first exon of the PGK1 gene, two HpaII sites previously shown to be differentially methylated, as well as the BstXI polymorphic site. Because PCR cannot maintain methylation patterns, the DNA samples were digested with HpaII (digestion was checked by the addition of λDNA) and the HpaII-digested, as well as undigested samples, were amplified. The amplified products were subsequently digested with BstXI and analyzed on gel. To facilitate the analysis of cell lysates (5,000 cells) a “nested”-PCR protocol was applied. After thawing of the samples, distilled water and buffer solution was added. Following EcoRI predigestion, samples were amplified for 15 cycles (0.5 minute, 95°C; 0.7 minute, 55°C; 0.7 minute, 72°C) using a set of “outer” primers (5 pmol each; ‘coding primer, GTG TGG GCC CTG TTC CTG CC; 3’ antisense primer, TAT CCT TTT GTG CAG GAA CC; amplicon length (±BstXI), 646/584 bp) in 100 μL buffer with a final concentration of 10 mmol/L Tris-HCl (pH 8.4), 1.5 mmol/L MgCl2, 50 mmol/L KCl, 0.02% BSA, and also consisting of 250 μmol/L of each dNTP, and 2.0 U of Taq polymerase (Cetus, Emeryville, CA). From this, 10-μL aliquots were subsequently amplified for 33 cycles using a set of “inner” primers (50 pmol each; 5’ coding primer, TGT TCC GCA TGC TGC AAG CC; 3’ antisense primer, TGT GAG ATG TGG GCC GTT TT; amplicon length (±BstXI), 598/557 bp). A clonal pattern is defined as the complete loss of one band in the HpaII predigested samples. In some cases, one of the bands in the HpaII predigested samples had a markedly reduced intensity, but remained visible. This pattern was designated “oligoclonal,” indicating either nonrandom X-inactivation or the presence of a mixture of both clonally and polyclonally derived cells. In the case of a polyclonal pattern, both bands in the HpaII predigested fractions exhibit two clear bands.

RESULTS

In total, 21 patients were screened for a polymorphism for one of the X-linked markers by Southern blot analysis of DNA derived from PB or BM cells. Six (29%) and two (10%) patients, respectively, were polymorphic for the PGK1 or the HPRT gene. Ten of 13 (77%) patients were informative for the M27P probe. Clonality was studied in 11 patients (Table 1). A monoclonal pattern was found in PB and/or BM cells in all patients tested (Table 2). One patient (UPN 06) was analyzed with two probes (PGK1 and M27P) and one patient (UPN 08) with all three probes. In both cases, a clonal pattern was seen, irrespective of the probe used. Purified cell suspensions of PMN, monocytes, lymphocytes, and/or T cells were prepared from PB obtained from UPN 01, 04, and 05 heterozygous for the PGK1 gene. A clonal pattern was found in myeloid (PMN, monocytes), but not in the lymphoid cells. Representative Southern blots hybridized with the PGK1, HPRT, or M27P probes are presented in the Figs 1 through 3.

The sensitivity of PCR analysis of the PGK1 gene was determined by titrating DNA obtained from a healthy female control, heterozygous for the PGK1 gene, against DNA derived from clonal PMN of a heterozygous patient. Polyclonally derived DNA could be detected in samples containing 5% to 10% normal control DNA (Fig 4). PCR analysis was subsequently performed on PB and BM cells and on purified PMN and lysates of FACS-sorted mono-
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Fig 2. Southern blot of UPN 08 showing a monoclonal pattern in PB and BM cells after hybridization with a probe for the HPRT gene. Lanes 1 show the BamHI polymorphism after PvuII and BamHI digestion, lanes 2 show the results of subsequent HpaII digestion. (A) Hybridized with pHPR-600, showing loss of polymorphism after HpaII digestion (type II inactivation pattern); (B) hybridized with pHPR-800. The upper bands in lanes 2 (B) are shorter because of HpaII digestion at sites 2 and 3 of the active allele. (For detailed information: see text and ref 19). The length of the fragments is given in kilobases.

cytes, T and B lymphocytes, and NK cells of UPN 01, 04, 05, 08, and 09, heterozygous for the PGK1 gene (Table 3). PB and BM cells showed a nonrandom, unilateral pattern, compatible with a mixture of clonally and polyclonally derived cells. In accordance with the Southern data, cells of myeloid origin (PMN and monocytes) appeared to be clonally derived, whereas circulating T and B lymphocytes, and NK cells exhibited a random X-chromosome inactivation compatible with a polyclonal pattern. Analysis of T-cell subsets (CD3+CD45RA+ and CD3+CD45RO+) also showed a random X-inactivation pattern. From UPN 01, 04, 05, and 08 also constitutive DNA derived from either paraffin-embedded skin biopsy specimen, a mouth wash, or hair roots were available. In UPN 05, analysis of this constitutive DNA suggested the presence of skewed methylation with the lower band of the sample amplified after HpaII digestion appearing to be stronger than the upper band. In contrast, the lymphocytes of UPN 05, the upper band is either of equal or greater intensity as compared with the lower band in the HpaII precut cell samples (Fig 5). Therefore, we cannot rule out the presence of clonal lymphocytes against a background of polyclonal lymphoid cells in this patient.

DISCUSSION

This study shows the clonal nature of hematopoiesis in patients with MDS by analysis of X-chromosome inactivation patterns. In all cases tested, either by Southern blotting or by PCR analysis of the PGK1 gene, it was shown that myeloid cells are of clonial origin, whereas circulating T and B lymphocytes and NK cells exhibit a random pattern of X-chromosome inactivation. Because lymphocytes are long-living cells, it is unclear whether they were derived from unaffected progenitor cells or that their origin had antedated the onset of MDS. We have considered the possibility that memory T cells were of polyclonal origin and that naive T cells were clonally derived. However, a polyclonal pattern was found in both subsets of cells. We also found a polyclonal pattern in lymphoid cells of patients with a long history of myelodysplasia (up to 104 months after diagnosis). However, we cannot rule out the presence of small numbers of clonally derived lymphocytes. In one of the patients analyzed, analysis of constitutive DNA suggested the presence of extreme lyonization in contrast to the pattern found in the lymphoid cells. Using G-6-PD isoenzyme analysis and X-linked RFLPs, clonal hematopoiesis has been consistently found in pa-
patients with MDS, but data are ambiguous with regard to lineage involvement. These conflicting results may not only be attributable to differences in disease duration in relation to lymphocytic longevity, but may also reflect heterogeneity of stem cell involvement in MDS. Also, in some studies purified lymphocytes were not available, and clonal lymphopoiesis was inferred from a skewed G-6-PD A:B ratio in Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines, or from a clonal pattern found in blood samples consisting of at least 40% lymphocytes. With a single exception, cytogenetic abnormalities have not been found in phytohemagglutinin-stimulated T cells of patients with MDS, nor in pokeweed mitogen-stimulated or EBV-transformed B cells. Chromosomal loss also was not detected using chromosome 7-specific DNA probes in lymphocytes of MDS and acute myeloid leukemia patients with monosomy for all or part of chromosome 7. We have recently shown cytogenetic involvement of myeloid cells, but not of lymphoid cells, using combined immunophenotyping and DNA in situ hybridization. Also, we have shown myeloid but not lymphoid involvement in fluorescence-sorted cells that have a 5q- anomaly using PCR amplification of VNTRs in hematopoietic growth factor and receptor genes located on the long arm of chromosome 5. These data are in accordance with data from a recent study of Abrahamson et al showing clonality of granulocytes and polyclonality of T and B lymphocytes in patients with RA and a 5q- anomaly or monosomy 7, using a combined approach of gene loss and X-linked RFLPs. In contrast to the data obtained by cytogenetic analysis, point mutations of the ras proto-oncogene have been shown to affect cells from both myeloid and lymphoid origin in patients with MDS.

Using X-linked RFLPs for clonality studies, caution should be exercised in the interpretation of data obtained from any one individual. Recent studies suggest that the incidence of skewed methylation is higher than previously reported, and might even be as high as 23%. In the present study, a nonrandom methylation pattern was found in all patients. The random methylation pattern found in lymphocytes served as control in five patients. In four of them we were able to analyze constitutive DNA derived from a mouth wash, hair roots, or paraffin-embedded skin biopsy specimen by PCR analysis of the PGK1 gene, showing a random X-inactivation in three patients, whereas a skewed lyonization was suggested in one.

PCR analysis of the methylation pattern of the PGK1 gene is a simple and rapid method to study clonality, showing results concordant with results of Southern analysis. PCR analysis was shown to be more sensitive than Southern blotting, because the mixture of clonally and polyclonally derived cells in PB and BM could be detected by PCR, but not by Southern analysis. Also, the contamination of PMN by polyclonally derived lymphocytes was detected in UPN 01 and 08 by PCR, but not by Southern analysis. Adaptation of this method made application feasible in clonality studies on limited cell numbers, ie, fluorescence-sorted cells that have a low frequency in peripheral blood. Gilliland et al have applied a similar technique to analyze cells present in individual hematopoietic colon...
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Clonal origin of PMN in patients with MDS and poly-cythemia vera can be determined by PCR because the methylation sites after clonal origin of PMN in patients with MDS and polymorphism of BstXI site are in close proximity and the methylation pattern of the gene is simple, in contrast to the HPRT gene and the DXS255 locus recognized by the M27β probe. However, the percentage of women heterozygous for the PGK1 gene is relatively low (about 33%).

In conclusion, some patients, MDS represents a disorder with clonal hematopoiesis in cells of myeloid origin, whereas a random X-inactivation pattern is found in circulating T and B lymphocytes, and NK cells. PCR analysis of the methylation pattern of the PGK1 gene is a simple and rapid method for analysis of clonality on limited cell numbers, and provides a tool to study highly purified subsets of cells.

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Clonal involvement of granulocytes and monocytes, but not of T and B lymphocytes and natural killer cells in patients with myelodysplasia: analysis by X-linked restriction fragment length polymorphisms and polymerase chain reaction of the phosphoglycerate kinase gene

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