Ann Hematol (1997) 74:193–197

CASE REPORT

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Myelodysplastic syndrome/acute myeloid leukemia supervening previously untreated chronic B-lymphocytic leukemia: demonstration of the concomitant presence of two different malignant clones by immunologic and molecular analysis

Received: 30 October 1996 / Accepted: 8 January 1997

Abstract The development of myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML) has rarely been observed in patients with chronic B-lymphocytic leukemia (B-CLL). So far, the discussion concerning the pathogenesis of the simultaneous occurrence of these two malignancies has been speculative, opposing the theory of two separate malignant clones to the theory of a common stem cell malignancy. We describe the case of a 77-year-old woman who developed MDS after 8 years of an indolent course of B-CLL. The diagnosis of MDS was based on bone marrow (BM) morphology, showing the typical picture of a refractory anemia with excess of blasts (RAEB). The clinical course of MDS was aggressive, terminating in AML within only 6 months. Immunophenotyping of BM and peripheral blood (PB) cells revealed a $CD34^+/CD13^+/CD33^-/CD19^-$ blast cell population and a $CD19^+/CD5^+$ B-cell population with kappa light chain restriction. Molecular analysis of PB and BM demonstrated the presence of an immunoglobulin heavy chain (IgH) gene rearrangement by polymerase chain reaction (PCR) amplification of genomic DNA with three different pairs of consensus primers. Cell-sorting experiments showed that the IgH gene rearrangement was present only in the $CD19^+/CD34^-$ B-cell population, but not in the $CD34^+/CD19^-$ blast cells. Furthermore, X-chromosome inactivation pattern analysis revealed two differently methylated cell populations. These experiments demonstrate the concomitant existence of two different clones in a patient with CLL-MDS/AML.

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Key words Chronic B-lympohocytic leukemia · Secondary MDS/AML · Clonal analysis · IgH gene rearrangement · DXS 255/M27β

Introduction

The development of myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) is a rare event in the course of chronic B-lymphocytic leukemia (B-CLL) [2, 4, 5, 11, 13–15, 20, 23]. Theories on the etiology of this phenomenon include chance coincidence, a CLL-associated immunologic defect [2, 23], previous treatment of CLL with alkylating agents [5, 20], and a common leukemogenic agent [20]. Some authors propose the theory of two separate malignant clones, whereas several authors also discuss evolution from a single clone [4, 11, 13, 14, 20]. So far, both theories have been based on morphological and immunologic grounds, but they have not been confirmed by cytogenetic or molecular studies. We present a patient with MDS/AML supervening previously untreated B-CLL and demonstrate the concomitant presence of two separate malignant clones using immunologic and molecular analysis.

Materials and methods

Case history

In December 1986, a 69-year-old woman was introduced to a hematology department because of leukocytosis. Her past medical history had been unremarkable except for a 10-year history of spondylarthrosis. At presentation, her white blood cell count (WBC) was 15.8 G/l, her differential count revealed 80% lymphocytes, red blood cell (RBC) and platelet (PLT) counts were normal, and there was no evidence of organomegaly. A bone marrow aspirate showed an infiltration with 50% of mature lymphoid cells. Based on these findings, the diagnosis of CLL was established. Because of the indolent stage of the disease, no antileukemic treatment was initiated. During the following 7 years, the WBC slowly increased to 40 G/l. RBC and PLT counts remained

normal, and organomegaly was absent. In October 1994, the patient presented for the first time with a normochromic anemia. The hemoglobin level was 10.7 g/dl; the WBC was 41.9 G/l; the WBC differential count showed 93% lymphocytes, 6% neutrophils, and 1% bands; the PLT count was 270 G/l; the physical examination was unremarkable. The levels of serum iron and ferritin were slightly elevated. A hemolytic process was excluded because of the absence of reticulocytosis and because of normal levels of lactate dehydrogenase (LDH) and haptoglobin. During the following months the anemia constantly worsened, whereas the WBC constantly increased. In December 1994, the hemoglobin level had dropped to 8.2 g/dl, the WBC count was 153 G/l, and the PLT count was 229 G/l. The spleen tip was palpable on physical examination. The WBC differential count revealed 92% lymphocytes, 4% neutrophils, 2% metamyelocytes, 1% myelocytes, and 1% blasts. The severe anemia was interpreted as a consequence of progression of CLL, and antileukemic treatment with chlorambucil was initiated. Consequently, the WBC decreased to 30 G/l within 1 month. The severe anemia remained unchanged, however. After discontinuation of chlorambucil therapy the WBC transiently increased, but it finally remained stable between 30 and 50 G/l without further cytoreductive treatment. The WBC differential count persistently revealed myeloid precursor and occasional blast cells. The severe anemia was treated with regular transfusions of packed red cells. A bone marrow (BM) aspirate was performed in April 1995. It revealed a hypercellular marrow with predominance of a dysplastic myelopoesis and a blast cell count of 5%. Erythroid precursor cells accounted for only 2% of nucleated cells. The percentage of mature lymphoid cells was 15%. Three months later, another BM aspirate showed an increase of blast cells to 11%, the percentage of lymphoid cells was 11%. Based on these findings, the diagnosis of refractory anemia with excess of blasts (RAEB) supervening B-CLL was established. The patient continued to receive regular transfusions of packed red cells. In September 1995, she developed overt AML with a peripheral blast cell count of 50%. Due to her poor physical condition, the patient did not receive any antileukemic chemotherapy, and she died only 1 month later of septic complications.

Morphological classification

Peripheral blood (PB) and BM smears were stained using a modified Wright technique. A total of 200 cells from the PB and a total of 500 cells from the BM were counted to establish the respective differential counts. Diagnoses of CLL and FAB classification of MDS were performed according to standard criteria [3, 16].

Immunologic phenotyping

PB and BM mononuclear cells (MNC) were prepared by Ficoll density (1.077) gradient centrifugation. MNC were stained with monoclonal antibodies (mAbs) using direct and indirect immunofluorescence techniques. Lymphocytes were characterized by a panel of B-lymphoid-associated mAbs: HD37 (CD19), DK23 (CD5), anti-surface IgM (S-IgM), DFT-1 (CD43) (DAKO, Glostrup, Denmark); VIL-A1 (CD10), HD39 (CD22) (Behring, Marburg/Lahn, Germany); IOB8 (CD23) (Immunotech, Marseille, France); FMC 7 (SERA LAB, Sussex, UK); anti CD19R/kappa, anti CD19R/lambda (TAGO, Burlingame, Calif.). The blast cells were characterized by mAbs MY10 (CD34), MY7 (CD13), MY9 (CD33) (Coulter, Hialeah, Fla.); MPO/LF (intracytoplasmic anti-myeloperoxidase/lactoferrin) (AN DER GRUB, Kaumberg, Austria); HD37 (CD19), DK23 (CD5). Dual staining experiments with mAb combinations to antigens CD19/CD34, CD19/CD5, CD34/CD13, CD34/CD33, CD19/CD13 and CD19/CD33 were performed to discriminate different cell types. The stained MNC were analyzed using a FACScan (Becton Dickinson, Mountain View, Calif., USA).

Cell sorting

After immunologic characterization, further specimens of PB and BM MNC were sorted on a FACStar Plus (Becton Dickinson) as previously described [9]. Double staining with mAbs HD37 (CD19) and HPCA2 (CD34) (Becton Dickinson) was used to enrich for $CD19^+/CD34^-$ lymphocytes and for $CD34^+/CD19^-$ blast cells. The purity of each sort was >98% as assessed by reanalysis of the sorted cell populations.

Molecular analysis

Analysis of immunoglobulin heavy chain (IgH) gene rearrangement. Polymerase chain reaction (PCR) analysis of the IgH gene rearrangement was performed on PB and BM MNC, as well as on sorted cells. Briefly, MNC were separated from BM or PB by density gradient centrifugation using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Genomic DNA was extracted from MNC using standard phenol-chloroform methods. DNA from sorted cell fractions was obtained by freezing, thawing, and boiling the cell suspension (for 10 min) followed by centrifugation at 12000 g (for 10 min). The PCR amplification was performed using three different pairs of consensus primers (VH26/OL-4 [12], V_H/J_H [19], and FR3A/VLJH [21]) as previously described. PCR products were electrophoresed through 6% polyacrylamide gels (Novex, San Diego, Calif., USA) and visualized by UV light after staining with ethidium bromide. In our laboratory this assay has a sensitivity of 1 rearranged cell in 10^2 normal cells. To exclude false-positive results, all experiments were performed twice and at least at two different timepoints. In order to avoid false-negative results in the presence of small numbers of neoplastic cells, dilutions of monoclonal DNA (1%) in polyclonal DNA isolated from PB of healthy volunteers were additionally amplified in each experiment to serve as a positive control.

Analysis of X-chromosome inactivation patterns. Clonal analysis of the DXS 255 locus was performed with PB MNC at the time of diagnosis of CLL/MDS (May 1995) and after evolution to AML (October 1995) as recently described [10]. Briefly, 30 μg of genomic DNA were digested to completion with the restriction enzyme *Pst*I. The DNA was divided into three aliquots. One aliquot was left unchanged, another aliquot was further digested with the methylation-insensitive enzyme *Msp*I, and the third aliquot was digested with the methylation-sensitive enzyme *Hpa*II, which cuts the recognition sequence (C/CGG) only when the internal C residue is not methylated and, thus, in case of the DXS 255 locus, identifies the inactive X-chromosome. Restriction enzymes were obtained from either Boehringer Mannheim (Mannheim, Germany) or Promega (Madison, Wis., USA) and were used at a concentration of 10 units/μg DNA under conditions recommended by the manufacturers. The samples were electrophoresed in 1% agarose gels and transferred to Hybond N (Amersham, Buckinghamshire, UK). Hybridization to probe M 27β and washing of the membranes was performed as described by Fey et al. [6]. Filters were exposed to Kodak X-omat-AR films (Eastman Kodak, Rochester, NY, USA) at −70 °C for 14 days. The critical limit for detection of clonal cell populations by this method is 40% of total cells [7].

Results

Morphology

During the phase of MDS, the peripheral blood smears constantly revealed marked lymphocytosis as well as a few myeloid precursor cells and occasional blast cells (Fig. 1a). The first bone marrow aspirate obtained in April 1995 showed a hyperplastic marrow with predom-

inance of a dysplastic myelopoesis and marked erythroid hypoplasia. The blast cell count was 5%, the lymphoid cell count was 15%. The second bone marrow aspirate from July 1995 showed an increase of blast cells to 11% (Fig. 1b). After progression to AML the peripheral blood cell count showed 50% of blast cells (Fig. 1c). The blast cells exhibited the morphology of FAB M1/M2 leukemia subtypes. On cytochemical stains they were positive for myeloperoxidase but negative for alpha naphthyl acetate esterase. The proportion of lymphoid cells was 18%. A bone marrow aspirate was not performed at that time.

Immunologic phenotype

Peripheral blood. Immunologic phenotyping of PB in April 1995 revealed a B-cell population accounting for 80% of total lymphoid cells. The cells exhibited the following phenotype: $CD19^+$, $CD5^+$, $CD23^+$, $CD43^+$, S-$IgM^{(dim+)}$, kappa light $chain^{(dim+)}$, $CD10^-$, $CD22^-$ and $FMC7^-$, lambda light $chain^-$. Positivity with anti-CD34 was found in 0.4% of nucleated cells. In September 1995 the blast cell count was 50%. The blasts exhibited the phenotype: $CD34^+$, MPO^+, $CD13^+$, $CD33^-$, $CD19^-$, $CD5^-$. The $CD19^+/CD5^+$ B-cell population was further demonstrable.

Bone marrow. Ninety percent of bone marrow lymphocytes were found to be B cells exhibiting the same phenotype as the B cells in the PB. Four percent (April 1995) and 7% (July 1995) of MNC were positive for CD34. The $CD34^+$ cells co-expressed CD13 and were negative for CD33 and CD19.

Cytogenetics

Cytogenetic analysis performed from BM during the phase of CLL/MDS and from PB after progression to AML revealed a normal 46XX karyotype on both occasions.

Molecular analysis

Analysis of IgH gene rearrangement. PCR amplification of genomic DNA with three different pairs of consensus primers revealed the presence of an IgH gene rearrangement in PB and BM MNC. Cell-sorting experiments of PB as well as BM during the phase of CLL/MDS showed that the IgH gene rearrangement was present in the $CD19^+/CD34^-$ B-cell population but not in the $CD34^+/CD19^-$ blast cells (Fig. 2a).

Analysis of X-chromosome inactivation patterns. PB MNC were shown to be clonal during the phase of CLL/MDS as well as after progression to AML, but with a different allelic pattern. In the CLL/MDS sample (WBC differential count: 75% lymphocytes, 2% blast cells), both alleles were fully methylated with no *Hpa*II digestion. In the AML sample (WBC differential count: 18% lymphocytes, 50% blast cells), a monoclonal picture was obtained with complete digestion of the unmethylated, inactive, low-molecular-weight allele and no digestion of the methylated, active, high-molecular-weight allele (Fig. 2b).

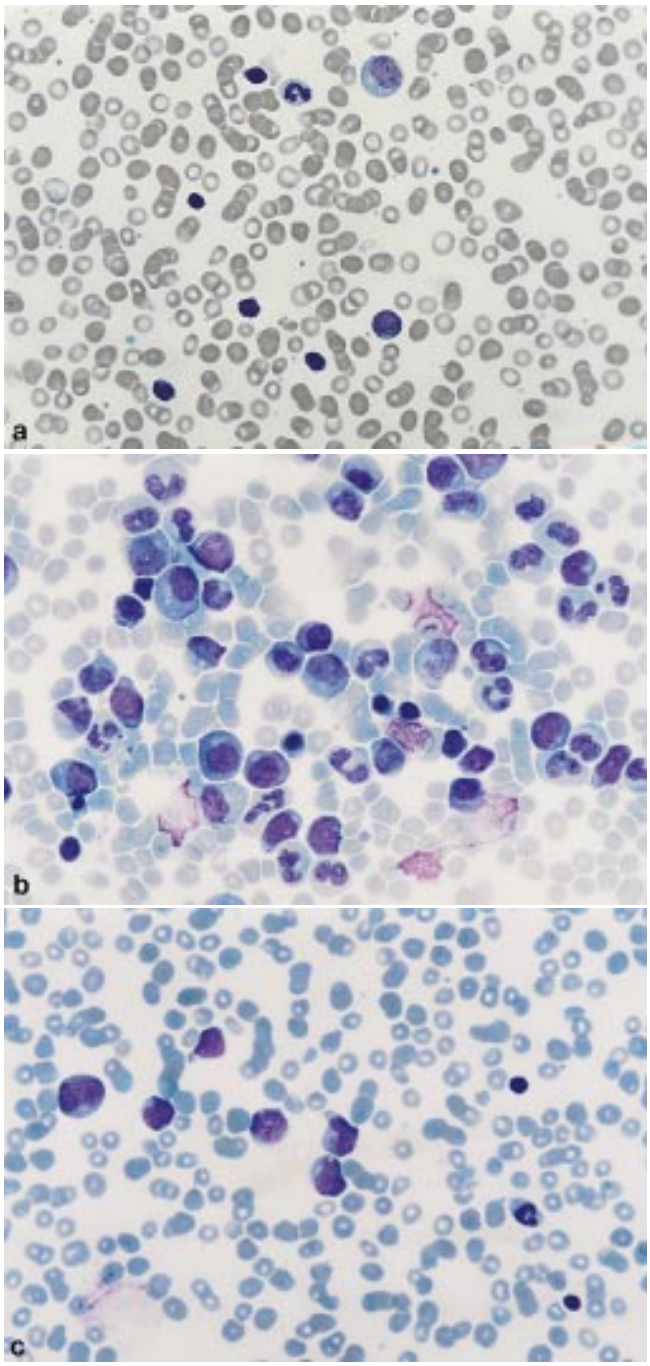

Fig. 1 Morphological findings of peripheral blood (**a**) and bone marrow (**b**) at the time of diagnosis of MDS; **c** peripheral blood smear after evolution to AML (modified Wright stain; original magnification ×60)

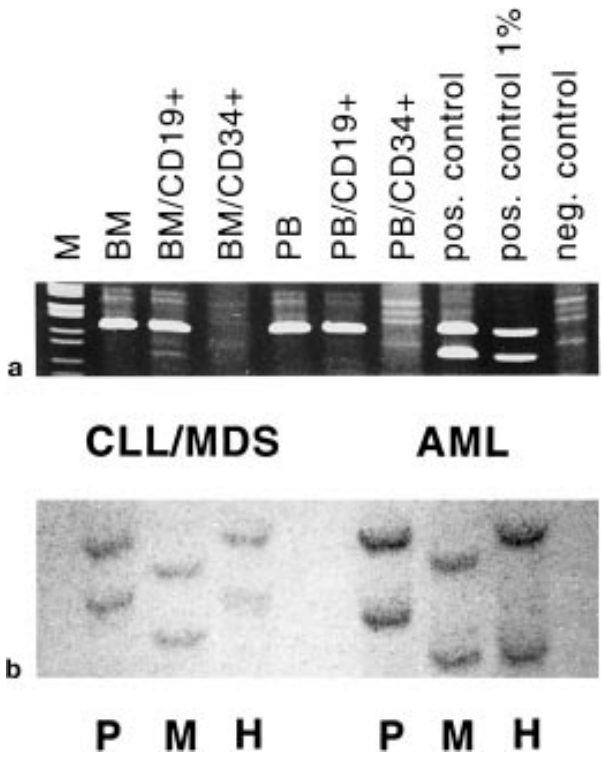

Fig. 2 **a** PCR amplification of the IgH gene rearrangement with consensus primers FR3A and VLJH. The rearrangement was detected in unsorted bone marrow (*BM*) and peripheral blood (*PB*) MNC and in the $CD19^+/CD34^-$ B-cell population, but not in the $CD34^+/CD19^-$ blast cell population. *M* Molecular weight marker; *positive control* undiluted and diluted (1%) DNA from another CLL patient; *negative control* healthy person. **b** Clonal analysis of peripheral blood MNC with M27β at diagnosis of CLL/MDS and after evolution to AML. The DNA samples were digested with restriction enzymes *Pst*I (*P*), *Pst*I + *Msp*I (*M*), or *Pst*I + *Hpa*II (*H*). In the CLL/MDS sample both alleles remained undigested with *Hpa*II, the AML sample showed a typical clonal pattern with complete digestion of the smaller allele

Discussion

The present case demonstrates the concomitant existence of two different malignant clones in a patient who developed MDS/AML after 8 years of an indolent course of B-CLL. The occurrence of the second malignancy was characterized (a) clinically, by the development of a severe anemia; (b) morphologically, by the emergence of myeloid precursor cells and blast cells in the peripheral blood, as well as the typical picture of MDS/RAEB in the bone marrow; (c) immunologically, by the appearance of a $CD34^+/CD13^+/CD19^-$ myeloid blast cell population besides the $CD19^+/CD5^+/CD34^-$ B-cell population; and (d) genetically, by the detection of an IgH gene rearrangement negative blast cell population in addition to the pre-existing IgH gene rearrangement positive B-cell clone, and further by the demonstration of two differently methylated clones using X-chromosome inactivation analysis. The pathophysiology of the concomitant occurrence of the two malignancies in the particular patient has to remain open. A chance coincidence seems likely, since both malignancies typically occur with advanced age. We cannot rule out a contributory role of a CLL-associated immunologic deficiency for the development of MDS/AML. Patients with CLL do have an increased incidence of secondary cancers [22], which might also explain the emergence of aberrant myeloid clones. A recent study, however, did not provide any evidence for an increased risk of AML/MDS in a cohort of more than 1000 patients with CLL [15]. Secondary MDS/AML as a consequence of treatment with alkylating agents [5, 20] can be ruled out in our patient, since the first clinical and morphological signs of MDS occurred shortly before the initiation of chlorambucil therapy, and the interval between the first administration of an alkylating agent and the definite diagnosis of MDS was only 4 months. Evolution of the two malignancies from a single clone can be excluded on the basis of the molecular genetic data. The rearrangement of the IgH gene is typically observed in B-lymphoid malignancies [21]. However, the IgH gene rearrangement has also been observed in about 15% of either acute T-lymphoblastic and acute nonlymphocytic leukemias [1, 17]. In the present case, the IgH gene rearrangement was demonstrable in the $CD19^+/CD34^-$ B-cell population, but it was absent in the $CD34^+/CD19^-$ blast cell clone. The presence of two different clones was further demonstrated by analysis of the X-chromosome inactivation pattern. Two separate analyses performed after diagnosis of MDS and after evolution to AML exhibited two genotypically different clones. The CLL/MDS sample showed two fully methylated alleles, a pattern which is often noticed in leukemias and lymphomas, but which is never seen in healthy persons [7]. The CLL/AML sample showed a typical monoclonal pattern. These results are explained by the low sensitivity of the assay [7], which detected only the lymphoid but not the myeloid clone during the phase of CLL/MDS, and only the myeloid but not the lymphoid clone after progression to AML. The different allelic patterns of the two malignant clones preclude the possibility that both clonal populations were derived from the same progenitor cell. Analysis of X-chromosome inactivation patterns might possibly help to further clarify the origin of second B-cell malignancies occurring in women with CLL [8, 18].

Although we can rule out a common stem cell malignancy in our particular patient, the leukemogenic factor(s) might have been the same for both leukemias. The patient's history was negative for any exposure to known leukemogenic agents. Extensive epidemiological studies would be helpful to better understand the pathogenesis of the concomitant development of hematological malignancies in a single patient.

Acknowledgements The authors want to thank Elvira Kitzweger, Dieter Printz, Helmut Sommer and Karin Winkler for their skillful technical assistance.

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