Myeloperoxidase Gene Expression in Blast Cells With a Lymphoid Phenotype in Cases of Acute Lymphoblastic Leukemia

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Many reports provided evidence of the presence of markers normally found only on cells of a different lineage in leukemic cells of a phenotypically well-defined lineage. Most of these cases are represented by cell populations with myeloid or monocytoid phenotypes that bear B- or T-lymphoid markers, either molecularly or immunologically detected (for example, Ig gene rearrangement in myeloid blasts, either molecularly or immunologically identified as lymphoid by immunologic and molecular criteria. In particular, the Ig heavy-chain gene rearrangement was largely prevalent, and the germ line configuration was almost absent. However, in three of eight cases, high levels of MPO mRNA were detected. The remarkable homogeneity of the cell populations examined suggests that the MPO mRNA observed was present in cellular elements certainly identified as lymphoid. The absence of contamination by myeloid cells was confirmed by the results of Western blot analysis of the proteins of the cell population studied: no MPO protein was detectable. The levels of MPO mRNA observed were high enough to be comparable to those observed in a promyelocytic cell population.

An alternative explanation has been proposed that is consistent with the existence of a pre-T/myeloid progenitor committed to the myeloid line. This hypothesis is supported by the following observations: (1) the absence of immunologic definition of the leukemic phenotype; (2) the presence of myeloid enzymes in cells with a well-characterized lymphoid phenotype has been described only in a few controversial cases; and (3) observations of this type have led to the concept of lineage infidelity of gene expression and may carry important implications for both leukemogenesis and normal differentiation. An alternative explanation has been proposed that is based on the evidence thus far presented that misprogramming of differentiation is at least partially unconvincing. As pointed out by Greaves et al., it is premature to presume that all genes whose activity is normally associated with particular individual lineages will be expressed exclusively and in all-or-nothing fashion. It is a common conviction that the use of new molecular methods will clarify the issue. The availability of a molecular probe for the myeloperoxidase (MPO) gene prompted us to investigate the levels of expression of this gene in several homogeneous blast cell populations clearly identified as lymphoid by immunologic and molecular criteria. An unexpectedly high level of the MPO mRNA was detected in three of eight patients in whom no MPO protein was detected by Western blotting. In our opinion, this finding indicates a condition in which gene expression at the mRNA level does not influence the phenotype characteristics and suggests that extreme caution should be used in reducing conditions of gene regulation from data obtained by immunologic definition of the leukemic phenotype.

MATERIALS AND METHODS

Cell characterization. The blast cell populations were obtained by leukapheresis. Phytohemagglutinin (PHA)-stimulated lymphocytes were obtained as already described. Morphologic evaluation was performed on May-Grünwald-Giemsa-stained smears. The following cytochemical reactions were performed: PAS, Sudan black B, diaminobenzidine, MPO, and alphanaphthol acetate esterase. Surface marker analysis was performed by indirect immunofluorescence using the following monoclonal antibodies: J5 (CD10), anti-B4 (CD19), anti-HLA-DR, MY4 (CDw14), MY7 (CDw13), MY9 (CDw3), OKT3 (CD3), OKT4 (CD4), OKT8 (CD8), and OKT16.

Molecular probes. The DNA fragments obtained from the plasmids carrying the gene probes used in this study were a 2.2-kilobase (kb) Eco RI-HindIII fragment from the human MPO cDNA as described by Johnson et al. and kindly provided by G. Rovera; a 1.2-kb PstI fragment from the human c-myc cDNA as described by Watt et al., also a generous gift of G. Rovera; a 2.1-kb BamHI fragment from the human β-actin cDNA as described by Gunning et al.; a 2.1-kb EcoRI genomic fragment from the histone H3 gene, a generous gift from G. Stiles; a 1.2-kb EcoRI subgenomic fragment containing part of the first and all the second exon of the heavy-chain gene as described by Ravetch et al.; and a 6-kb EcoRI genomic fragment including J-H sequences, as described by Ravetch et al. The different fragments obtained by digestion with restriction endonucleases were separated by polyacrylamide gel electrophoresis and recovered by using the electroelution procedure.

DNA labeling. All the purified DNA fragments were labeled by using the random prime labeling procedure described by Feinberg and Vogelstein. The specific activities obtained ranged from 2 to 3 × 10⁶ cpm/µg DNA.

Northern blot analysis. The total cellular RNA was extracted from the blast cells and from PHA-stimulated peripheral lymphocytes by using the phenol-chloroform method as described by Ferrari et al. Ten micrograms of RNA for each lane was loaded onto a 1.1% agarose/formaldehyde gel in 1 × MOPS buffer.

The RNA was transferred to Hybond (Amersham, Buckinghamshire, England) membrane by using the electroblotting procedure. After UV fixation of the transferred RNA, the membrane was prehybridized, hybridized with 32P-labeled probes as already described. The remarkable homogeneity of the cell populations examined suggests that the MPO mRNA observed was present in cellular elements certainly identified as lymphoid. The absence of contamination by myeloid cells was confirmed by the results of Western blot analysis of the proteins of the cell population studied: no MPO protein was detectable. The levels of MPO mRNA observed were high enough to be comparable to those observed in a promyelocytic cell population.
control. All the cell pellets (from 5 described by Burnette with minor modifications. Briefly, HL-60 to Gene Screen membrane (NEN Research Products, Boston). After digested with different restriction endonucleases (see the legends to dissolved in sterile TE buffer and stored at 4°C. The DNA was digested with proteinase K. Several more extractions were performed with phenol-chloroform/isoamyl alcohol. Ethanol-precipitated DNA was dissolved in sterile TE buffer and stored at 4°C for several hours. The autoradiography was performed by using intensifying screens at ~80°C. The films were analyzed by using a soft laser densitometer scanner.

**Southern blot analysis.** Southern blot analysis was performed as described by Southern. The cells were washed and lysed, and the DNA was extracted with buffer-saturated phenol. The supernatant was digested with proteinase K; the DNA was extracted again with buffer-saturated phenol and digested with ribonuclease A and then with proteinase K. Several more extractions were performed with phenol-chloroform/isoamyl alcohol. Ethanol-precipitated DNA was dissolved in sterile TE buffer and stored at 4°C. The DNA was digested with different restriction endonucleases (see the legends to Figs 2 and 3), size-fractionated on 0.8% agarose gel, and transferred to Gene Screen membrane (NEN Research Products, Boston). After prehybridization, the filters were hybridized with the difference 32P-labeled probes as already described by Torelli et al. After washing in 0.2 × SSC and 0.5% SDS at 80°C, the filters were exposed to Kodak film for 18 hours. As a molecular weight marker, we have used a mixture of 

**Western blot analysis.** Western blot analysis was performed as described by Burnette with minor modifications. Briefly, HL-60 cells, stored in liquid nitrogen and freshly prepared, were used as a control. All the cell pellets (from 5 to 10 × 10^6 cells) were lysed in TBS buffer (40 mmol/L Tris-HCl, pH 8.1, 150 mmol/L NaCl, and 5 mmol/L EDTA) containing 1 mmol/L phenylmethylsulfonylfluoride by three cycles of sonication at 30 seconds each. The lysates were centrifuged for one hour at 4°C and 12,000 g. Of the recovered supernatant, the proteins were measured by the Folin procedure. One hundred micromicrograms of total protein, denatured by boiling for five minutes in 1× Laemmli mixture, was loaded on a 10% polyacrylamide gel. After electrophoretic separation, half of the gel was stained by using the silver staining procedure (Bio-Rad kit, Richmond, CA) to control the amount of protein in each sample, and the other half was electroblotted to a nitrocellulose membrane. The membrane was pretreated for two hours at room temperature in the following reaction buffer: 50 mmol/L Tris-HCl, pH 8.1, 150 mmol/L NaCl, 150 mmol/L CaCl2, 0.04% Ficoll 400, 1.5% bovine serum albumin (BSA, immunoblot grade), 0.04% Na azide, and 0.1% Nonidet P40. The antibody (kindly supplied by Dr A. Tabilio) was used at a dilution of 1:100 in the same reaction buffer for 4 hours at room temperature. After six ten-minute washes in reaction buffer without BSA, the membrane was incubated in reaction buffer containing 300,000 cpm/mL of 125I-labeled protein A (3.7 MBq/mL; Amersham). The incubation lasted overnight at room temperature. After six additional ten-minute washes in reaction buffer without BSA, the membrane was exposed to Kodak film for 18 hours at ~80°C. As a molecular weight marker, we have used a mixture of high- and low-range proteins supplied by BRR Laboratories, Gettysburg, MD.

### RESULTS

**Characterization of the blast population studied.** As shown in Table 1, the blast percentage was very high, ranging from 88% to 96% in all patients. The cytochemistry showed that a variable proportion of cells had a granular PAS positivity, whereas MPO activity was constantly detectable as was alkaline phosphatase esterase. The simultaneous assay in HL-60 cells was 98% positive, and in case of acute promyelocytic leukemia (APL) that was 95% positive, the Sudan black B reaction was completely negative. The immunologic characterization of the cells included 82% to 90% positive cells for CD10 and a lower proportion for anti-HLA-DR and CD19. CD3, CD4, CD8, and OKT16 were positive only in a small proportion of cells (3% to 8%). CDw13, CDw14, and MY9 (CDw33) were constantly negative. The molecular study of the genome showed that in all cases analyzed the Ig heavy-chain genes were rearranged.

**Northern blot analysis of MPO gene expression.** Figure 1 shows the results of Northern blot analysis of the total RNA extracted from the cells of the populations studied. The results of Northern blot analysis of the RNA extracted from blast cells of a promyelocytic leukemia and from PHA-stimulated normal peripheral lymphocytes are also shown. In samples 2, 4, and 8, two regions of hybridization were detected with the MPO probe at approximately 3.0 and 3.5 kb. The 3.5-kb band was clearly prevalent. To show evidence

![Fig 1. Northern blot analysis of the total cellular RNA extracted from the circulating blast cells of eight patients with ALL, lanes 1 to 8, one patient with APL, lane 9, and normal peripheral lymphocytes stimulated for 64 hours with PHA, lane 10.](from www.bloodjournal.org)
of differential expression of other genes, c-myc and histone H3 probes were also hybridized, and the results are shown in Fig 1. The variability of expression of these cell cycle–related genes has already been reported.23 The high level of expression of heavy-chain mRNA in all the acute lymphoblastic leukemia (ALL) populations studied is also evident in Fig 1. The RNA present in each lane was monitored by the β-actin mRNA levels.

**Southern blot analysis.** In seven of eight patients studied, Southern blot analysis of the DNA with different restriction endonucleases and the Ig heavy-chain probe showed that the rearranged allele was prevalent. Figure 2 shows a composite panel of autoradiographs of the Ig gene rearrangement in patients 2 and 4. Southern blot analysis was not carried out in patient 8 since the small amount of cells available allowed only the extraction of RNA and protein. Figure 3 shows the Southern blot analysis of DNA from patients 2 (B.R.) and 4 (P.G.) and from a normal subject (N) when using the MPO probe. The restriction pattern is in keeping with the results reported by Morishita et al.24 No rearrangement or amplification of the gene was observed.

**Western blot analysis.** In the three lymphoid blast cell populations expressing the MPO mRNA, the search for the MPO protein was completely negative. A simultaneous search for the MPO protein was highly positive in HL-60 cells as well as in a patient with APL. The same amount of protein was detected both in fresh and frozen HL-60 cells. In the positive cells, the prevalent molecular species was 55 Kd, but an 80-Kd and a 39-Kd species as well as other very faint bands were detectable. As shown in Fig 4 (panel B), the silver staining showed that approximately the same amount of protein was loaded for each lane.

**DISCUSSION**

We have reported evidence that in cell populations obtained from three ALL patients the MPO gene was expressed at a fairly high level. The lymphoid phenotype of these populations was determined by cytochemical, immunologic, and molecular criteria. The remarkable homogeneity of the results obtained by using various techniques with differing methodology to define the phenotype leads us to rule out the presence of more than a negligible fraction of myeloid cells, which is particularly confirmed by the complete lack of cells carrying myeloid antigens. The almost total absence of Ig heavy-chain genes in the germ line configuration is further evidence that these populations were of monoclonal origin. Our data taken as a whole indicate that the MPO mRNA was present in clonal populations unimpeachably characterized as lymphoid. At least some of the most common causes of inappropriate gene expression such as gene rearrangement or amplification can be ruled out in
our patients because neither gene rearrangement nor amplification was detected by Southern blot analysis. In our patients, the presence of a normal-sized MPO mRNA was accompanied by the lack of any detectable enzyme activity. This condition has already been described in the myeloid cells of subjects with a complete deficiency of MPO. However, in these cases, small amounts of MPO precursor peptides but not mature MPO peptides were detected. On the contrary, in our patients Western blot analysis did not show any evidence of either precursor or mature MPO peptides. The relatively high frequency (three of eight) of the persistence of the MPO mRNA that we have observed, at least in our limited series, suggests that this event may occur physiologically in the very early stages of lymphoid differentiation and that, because of clonal selection, it may remain evident in some lymphoid blast cell populations. As far as we know, this is the first time that a specific myeloid molecular probe has been used to explore the regulation of genome expression in lymphoid cells. Our results suggest that to explore the molecular mechanisms underlying cell differentiation in addition to studying the protein level, an investigation of the messenger RNA level, as suggested by Chang et al, is necessary.

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