CONCISE REPORT

Immunoglobulin Gene Rearrangements in Remission Bone Marrow Specimens From Patients With Acute Lymphoblastic Leukemia

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Recombinant DNA probes for the joining (JH) segment of the immunoglobulin heavy chain gene were used to detect molecular rearrangements of this gene in the DNA of bone marrow cells obtained during remission of acute lymphoblastic leukemia (ALL). This molecular approach was optimized and found to exceed the sensitivity of conventional morphologic screening for detecting residual leukemia cells; one leukemic cell in 500 normal nucleated bone marrow cells was easily detected using this approach. In the present study, bone marrow from three of seven patients in complete clinical remission (defined morphologically) contained leukemic cells in these proportions. This analysis may be of use in evaluating the status of clinical remission in selected ALL patients.

MATERIALS AND METHODS

Cells. Bone marrow aspirates were diluted 1:5 with RPMI-1640 and allowed to sediment at 1 g for one hour. Cells from the buffy coat and the floating spicules (released by repeated passage of the spicules through a 25G needle) were pooled and washed with RPMI-1640. Mononuclear cells were then purified on Histopaque 1.077 (Sigma, St Louis) step gradients. A minimum of 3 x 10^6 purified mononuclear cells was required for this analysis of rearranged Ig genes.

Hybridization analysis. DNA was purified by sodium dodecyl sulfate (SDS)-proteinase K digestion followed by phenol-chloroform extraction and DNA concentrations were determined by the diphenylamine method. DNA was cleaved consecutively with HindIII and BamHI restriction endonuclease obtained from Bethesda Research Laboratories (Bethesda, Md) using 10 U of enzyme per microgram of DNA in reaction buffers recommended by the supplier. Of the cleaved DNA, 7.5 µg were concentrated by ethanol precipitation and subjected to vertical agarose gel electrophoresis using a slot of 3 x 8 mm. Electrophoresis, transfer to nitrocellulose, and hybridization were performed as previously described. The BamHI-HindIII fragment of the JH region, cloned in plasmid pBR322, was provided by Dr Philip Leder. Nick translation of this plasmid clone was performed using the kit from Amer sham (Arlington Heights, Ill) following the supplier’s conditions but using 10 ng of DNA and 50 µCi of α³²P-dCTP (3,200 Ci/mmol) for one hour at 17 °C. Autoradiography was performed for 15 hours at –80 °C using Kodak XAR-5 film (Rochester, NY) and Dupont Lightning Plus intensifying screens (Dupont, Wilmington, Del).

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RESULTS

In order to optimize the detection of clonally derived cells containing identically rearranged Ig genes, we concentrated on two aspects of the analysis: methods of bone marrow preparation and nucleic acid hybridization techniques. It was of paramount importance that the bone marrow aspirate be drawn with minimal blood contamination, which could obviously dilute any leukemic cells present in the marrow with a much larger number of normal circulating cells. A two- to fivefold enrichment for mononuclear cells was achieved by techniques described in the Materials and Methods section.

Molecular biological parameters were optimized by implementing a model bone marrow population prepared by in vitro mixing of nonrearranged DNA from normal cells with varying amounts of clonally rearranged DNA from leukemic blast cells. One variable examined was the portion of the JH region that would produce a strong "signal" upon hybridization to the rearranged DNA fragment and at the same time yield a minimum of "noise" or background hybridization to other nonspecific sequences. In this regard, several subfragments of the JH region (Fig 1) of the Ig heavy chain gene were tested in hybridization. Fragments A through E, shown in Fig 1, which were produced by cleavage with the enzyme Cpf I (Worthington Diagnostics, Freehold, NJ), and the entire BamH1–HindIII fragment, were tested individually as hybridization probes. Fragments A and B did not detect most rearranged genes, fragment C yielded the same background but less signal than the total plasmid clone, and fragments D and E yielded reduced background but also reduced signal. Therefore, the 6 kb BamH1–HindIII fragment encompassing all the JH genes was used in further experiments.

Three different radiolabeling techniques of the cloned JH BamH1–HindIII fragment were compared. The conditions were varied to produce a probe with a high specific activity, a low degree of random hybridization or background, and a level of detection sufficient to detect <5% leukemic cells in a normal population. Synthesis of RNA from the JH probe using the SP6 polymerase system11 (Promega Biotech, Madison, Wisc) fulfilled the first two criteria but not the third. "Oligo-labeling," performed using random hexamers as primers,12 as well as nick translation produced probes of high sensitivity, but the background was somewhat less using the nick translated probe. A high specific activity (2.5 × 10^9 dpm/µg) using nick translation was obtained by using only 10 ng of the JH plasmid in a reaction mixture containing 50 µCi of α³²P-dCTP (3,200 Ci/mmol).

The choice of restriction endonuclease with which the genomic DNA was cleaved was varied to select a combination which would reveal rearrangement of JH sequences in a DNA fragment with a significantly different mobility from the normal nonrearranged DNA upon electrophoresis. A combination of BamH1 plus HindIII produced a 6 kb DNA fragment in a normal sample (nonrearranged DNA) corresponding to the complete JH probe while rearranged fragments in the leukemic samples were usually of significantly different size. Other enzyme combinations tested did not produce easily distinguishable, rearranged fragments from leukemic cells as frequently as the BamH1–HindIII combination. In addition, the BamH1–HindIII combination had optimal complementarity with the probe. Fig 2A shows a typical experiment in which rearranged DNA fragments were detected in DNA extracted from leukemic cells of patients during the active phase of their disease (presentation or relapse). In each case, one to three rearranged fragments were identified and usually a normal, 6 kb fragment was also observed, representing unarranged Ig alleles still remaining in the leukemic cells or nonleukemic cells present in the cell fractions used for analysis.

Using the optimized methods, mixing experiments were performed to demonstrate the limits of detection of leukemic cells in the assay. One leukemic cell in 333 normal cells could be clearly detected in the original autoradiograph (Fig 2B). The source of the normal cells (placenta or peripheral blood mononuclear cells) did not affect this sensitivity. Since the cell fractionation procedure resulted in a two- to fivefold enrichment of mononuclear cells from bone marrow, one could specifically detect, at minimum, one leukemic cell in 500 normal bone marrow cells.

We next examined actual remission bone marrow samples to determine how frequently leukemic cells could be detected using the recombinant DNA analysis. In each case, the bone marrow sample examined had been independently diagnosed as indicating complete clinical remission by morphologic methods. In Fig 3, patient No. 1 is illustrative of the results that can be achieved. This patient was a 25-year-old male who presented with ALL in Feb 1985 (Table 1). Clinical remission was achieved in May 1985 following treatment with prednisone and vincristine and then arabinoside-c (ara-c) with daunomycin given in a timed sequence. At the time of bone marrow recovery, an aspirate of the bone marrow was obtained. Maturation of all cell types was observed with normal numbers of blast cells (<5%). After fractionating the bone marrow to enrich for mononuclear cells, we analyzed the DNA of the cells for rearranged bands characteristic of the patient’s leukemic cells. In DNA from his leukemic cells (obtained at the time of initial disease presentation), two rearranged fragments of 3.5 kb and 2.5 kb were detected (Fig 3, lane L, patient No. 1). These same fragments were detected in the DNA from the clinical remission bone marrow (Fig 3, lane R, patient No. 1). Note that the relative intensity of the rearranged bands is much less in lane R than in lane L, even though lane R contained 7.5 µg of DNA and lane L contained only 0.5 µg of DNA. We estimated that the rearranged bands in the remission sample corresponded to 1% to 2% leukemic cells in this bone marrow. The prevalent band at 6 kb derives from normal cells in the bone marrow. The 4.3 kb fragment was an internal control for the hybridization reaction.

![Fig 1. Map of immunoglobulin JH region of the human genome. CPF-1 sites were mapped in the region described in ref. 3.](image-url)
Fig 2. (A) Rearranged Jγ fragments in patients with leukemia. DNA was extracted from ALL cells of six patients (A through F) during the active phase of their disease (presentation or relapse). As described in Materials and Methods, 0.5 to 2.0 μg of DNA was cleaved with BamH1 and HindIII, and these digests were used in electrophoresis, Southern transfer, and hybridization. An autoradiograph of the Southern blot is shown with sizes of fragments noted on the left in kilobases (kb). Patients A, C, and E had one rearranged allele and one deleted allele, while patients B, D, and F had patterns which indicated a different rearrangement for each of two alleles. (B) Mixing experiment as model of remission bone marrow. Placenta DNA and DNA from patient C in Fig 2A was digested with BamH1 and HindIII. The normal DNA was mixed with leukemic DNA at the indicated ratios representing 7.5 μg normal plus 2.25 μg leukemic (1:3.3 or 30%), 7.5 μg normal plus 1.12 μg leukemic (1:0.7 or 15%), 7.5 μg normal plus 0.563 μg leukemic (1:13.3 or 7.5%), 7.5 μg normal plus 0.225 μg leukemic (1:33 or 3%), 7.5 μg normal plus 0.113 μg leukemic (1:66 or 1.5%), 7.5 μg normal plus 56.3 ng leukemic (1:133 or 0.75%), 7.5 μg normal plus 22.5 ng leukemic (1:333 or 0.3%), and 7.5 μg normal plus 11.3 ng leukemic (1:666 or 0.15%), respectively. Also individually represented (lanes C and N, respectively) are 2.25 μgrams of leukemic cell DNA and 7.5 μg of normal DNA.

Similar analyses were performed on a total of seven patients (Table 1) in whom morphologically normal clinical remission bone marrows were obtained. Of these seven patients, three exhibited leukemic cells in the remission marrows assessed by the recombinant DNA method (ie, patients No. 1, 2, and 3 of Fig 3 and Table 1). Two controls were required for unambiguous interpretation of these results. First, DNA from the leukemic cells of the same patients was used as a positive control to ensure that the rearranged fragments observed in the remission samples corresponded to the fragments detected in the leukemic cells. Second, normal DNA from the peripheral blood granulocytes of the patient was included as a negative control to verify that any faint rearranged bands were not attributable to the patient’s normal genes. In Fig 3, patients No. 2 and 3 displayed rearranged fragments in their remission bone marrow samples (noted with asterisks in lanes R, R1, and R2) which were not present in the DNA from normal cells of these patients (lane G). In the R2 remission sample of patient No. 3, a third rearranged band was present that was not observed in the original leukemic cells or the granulocytes from this patient. This third fragment may have represented a further rearrangement of the immunoglobulin gene which occurred in a subfraction of the leukemic cells. In patients No. 2 and 3, the number of leukemic cells in the remission specimens was estimated as one in 500 and one in 200 nucleated marrow cells, respectively. Patient No. 4 is included to illustrate a clinical remission bone marrow sample with less than one leukemic cell in 500 normal cells as detected in our assay.

Fig 3. Rearranged DNA fragments of leukemic blasts in remission bone marrows of ALL patients. Each panel depicts a different patient’s samples. L denotes DNA from leukemic cells, either presentation or relapse. R denotes remission as defined by morphologic examination (<5% blast cells). Patient No. 3 had two separate remission episodes analyzed. G denotes DNA from the granulocytic cells (included as a negative control for DNA rearrangements). All lanes depict 7.5 μg of DNA digests except patient No. 1, lane L (0.5 μg), and patients No. 3 and 4, lane L (1 μg). Size is indicated at left in kilobases. All samples contained 1 pg pBR322 DNA as a control for hybridization and transfer efficiency. Dots indicate rearranged Jγ fragments present in the various samples.

DISCUSSION

Using recombinant DNA techniques described above, we have detected one leukemic cell among more than 500...
normal nucleated cells in remission bone marrows. In order for this technique to be useful in the analysis of remission bone marrows, a significant number of bone marrows must contain enough leukemic cells to be recognized by the recombinant DNA assay (more than one in 500) but less than sufficient for recognition by morphologic evaluation (1/20). In the initial analyses of the seven patients reported here, three of the bone marrow samples exhibited numbers of leukemic cells than sufficient for recognition by morphologic evaluation. In order to help in diagnosis of patients with questionable remission status as assessed morphologically and in patients in whom alternative therapy, such as autologous or allogeneic bone marrow transplantation, is being considered.

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**REFERENCES**


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