Heavy Chain Immunoglobulin Gene Rearrangement in Acute Nonlymphocytic Leukemia

By Ugo Rovigatti, Joseph Mirro, Geoffrey Kitchingman, Gary Dahl, Judith Ochs, Sharon Murphy, and Sanford Stass

Samples of leukemic cell DNA from 14 children with acute nonlymphocytic leukemia (ANLL) and 4 human myeloid leukemia cell lines were analyzed for rearrangement in the heavy chain region of the immunoglobulin gene. The diagnosis of ANLL was confirmed in all patients by morphologic, cytochemical, and immunologic studies. By restriction endonuclease digestion and hybridization with cloned heavy chain immunoglobulin gene probes for the constant (C) and joining (J) regions, the DNA of 2 patients and 1 cell line (ML-1) was found to contain rearrangements. The DNA from the remaining 12 patients and 3 cell lines was not rearranged (germline configuration). Both patients with apparent immunoglobulin gene rearrangement achieved complete remission on therapy for ANLL. Immunoglobulin gene rearrangement in phenotypically defined ANLL suggests (1) that such changes may not be limited to lymphoid leukemia of B cell lineage, or (2) that, in some patients, the leukemic transforming event may involve stem cells capable of both B cell and myeloid differentiation.

MATERIALS AND METHODS

Patients

Fourteen consecutive patients with ANLL, for whom adequate numbers of leukemic cells could be obtained after informed consent, were studied. This investigation was part of a protocol-controlled cell profile analysis approved by the institution’s Clinical Trials Committee.

Cytochemical Studies

Leukemic blasts from blood and bone marrow were studied with Wright's stain. Marrow smears were also stained with standard cytochemical reagents, including periodic acid-Schiff reagent (PAS), myeloperoxidase (MPO), alpha naphthyl butyrate esterase (ANB), and Sudan black B (SBB), as previously detailed. When >3% of blasts reacted, the test was considered positive.

Cell Surface Phenotyping

Leukemic cells were isolated and analyzed with a standard indirect immunofluorescence assay (IFA), as described previously. Only samples containing >80% leukemic blasts were tested, and samples were considered positive if >25% of the cells had ring fluorescence. All assays included isotypic myeloma immunoglobulin as negative controls.

Each case was assessed with monoclonal antibodies that identify cells of nonlymphoid lineage: anti-My-1, MCS-1, and SJ-D1.
Cell Lines

All cell lines were cultured in a humidified 37°C, 5% CO₂ atmosphere in medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (K.C. Biologicals, Kansas City, MO). The ML-1²³ and HL-60²² cell lines were cultured in RPMI 1640 medium, the KG-12³ cell line in MEM α-medium, and the K562⁴ cell line in DMEM medium.

Terminal Deoxynucleotidyl Transferase (TdT)

The TdT assay was performed by IFA as described previously,²⁵ except in patient 2, for whom a quantitative enzyme assay was also performed.²⁶

Immunoglobulin Gene Rearrangement

Leukemic cells were isolated, and DNA was extracted as previously described.²⁶ Ten micrograms of DNA from each patient was digested with the restriction enzyme BamHI (Amersham, Arlington Heights, IL) or HindIII (Amersham), 3 U/µg DNA. The digested DNA was fractionated by electrophoresis on a 0.8% agarose gel.²⁸

The DNA was transferred to nitrocellulose paper according to the technique of Southern and hybridized with nick-translated probes for cloned heavy-chain Ig genes with specific activities of 2-5 × 10⁶ cpm/Mg DNA. Our probe for the human M-chain constant region domain and portions of C₅ and C₇ 3' was obtained from C. M. Croce (Wistar Institute, Philadelphia, PA), and contained a 3.3 kb BamHI/EcoRI fragment subcloned into pBR322. The subcloned region included 2.2 kb of J region DNA and 1.1 kb of 3' flanking sequences. The regions represented by the two probes are indicated in Fig. 1A. High stringency conditions were used for hybridization and washing.²⁹

The filters were washed, dried, and exposed at -70°C to XAR-5 films (Kodak, Rochester, NY) for 1–5 days in the presence of intensifying screens. All experiments included control DNA extracted from mononuclear cells of normal volunteers; additional controls included DNA from normal tonsil or spleen.

RESULTS

In all 14 cases, the morphological features disclosed by Wright-staining were consistent with ANLL. The diagnoses were confirmed by cytochemical staining.²⁷ Studies with monoclonal antibodies specific for non-lymphoid surface antigens, performed for 13 of the 14 patients (the sample from no. 6 was inadequate for analysis), confirmed the surface phenotype as non-lymphoid (Table 1). Results of the TdT assay, available for 9 of the 14 patients, were negative in all but 1 case.

DNA from each patient was incubated with the restriction endonuclease BamHI. After fractionation by agarose gel electrophoresis, the DNA fragments were transferred to nitrocellulose paper and tested with the C₅-specific probe. Results for all 14 patients are shown in Table 1; those for 8 patients, including nos. 1 and 2, and the restriction map of the human heavy chain Ig region are shown in Fig. 1.

The DNA fragments from 6 patients (Fig. 1B, lanes 6–11) were indistinguishable from the germline DNA fragment of 17 kb. DNA digests from patient 1, however, demonstrated a band of approximately 16 kb that hybridized with the C₅ probe, in addition to the germline band of 17 kb (Fig. 1B, lanes 2 and 3). Furthermore, DNA from patient 2 had two rearranged fragments (Fig. 1B, lanes 4 and 5). One was judged to be approximately 18 kb; the other was approximately 14.5 kb. The DNA in lane 4 was obtained from peripheral blood mononuclear cells and that in lane 5 from bone marrow. On careful analysis of lane 4 (Fig. 1B), a faint band (4% of the total DNA by densitometry) corresponding to the germline configuration (17 kb) was evident. This fragment most likely reflects the

<table>
<thead>
<tr>
<th>Table 1. Laboratory Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>14</td>
</tr>
</tbody>
</table>

*Percentage of reactive cells by IFA determination.
†Quantitative enzyme assay.

NG, negative; —, <25%, reactive; ND, not done; M1, myelocytic leukemia without differentiation; M2, myelocytic leukemia with differentiation; M4, myelomonocytic leukemia; M5, monocytic leukemia.
IgG GENE REARRANGEMENT IN ANLL

Fig. 1. (A) Schematic representation of the human Ig gene region with restriction endonuclease sites: E = EcoRI, H = HindIII, and B = BamHI. The C\textsubscript{\gamma} probe\textsuperscript{\textsuperscript{32}} consisted of a 1.2-kb EcoRI fragment subcloned into pACYC184 and contains the complete C\textsubscript{\gamma} 2 domain and portions of C\textsubscript{\gamma} 1 and C\textsubscript{\gamma} 3. The J\textsubscript{\gamma} probe\textsuperscript{\textsuperscript{34}} consisted of a 3.3-kb BamHI/EcoRI fragment subcloned into pBR322 and contains 2.2 kb of J region DNA and 1.1 kb of 3' flanking sequences. (B) Southern blot analysis, with BamHI and the C\textsubscript{\gamma} specific probe, of DNA from 8 patients with ANLL. Representative results for patient 1 are shown in lanes 2 (peripheral blood) and 3 (bone marrow), and for patient 2 in lanes 4 (peripheral blood) and 5 (bone marrow). Bone marrow findings for patients 3, 6, 9, 10, 12, and 13 are presented in lanes 6–11, respectively. DNA from normal peripheral blood (lane 1) served as the control.

To verify these findings and the specificity of the C\textsubscript{\gamma} probe, we washed the same Southern blot filter and rehybridized using the J-specific probe.\textsuperscript{31} This probe identified the same molecular-weight DNA fragments in all patients and confirmed the heavy chain gene rearrangements in patients 1 and 2 (data not shown). The DNA extracted from patients 1 and 2 was also studied with the C\textsubscript{\gamma} probe after digestion with HindIII. These experiments also demonstrated that DNA from patient 1 contained one heavy chain Ig gene rearrangement and that from patient 2 contained two (data not shown).

The rearranged band, of approximately 16 kb in patient 1, was more intense than the germline band (Fig. 1B). This may indicate the presence of a minor subclone of leukemic cells with germline Ig gene configuration, or one rearranged and one deleted allele with the germline contribution from normal cells. The Wright-stained peripheral blood sample in lane 2 contained 20% normal cells. Importantly, the leukemic cells of patient 1 did not demonstrate more than two chromosome no. 14s. DNA isolated from peripheral blood cells of patient 2, while she was in complete remission, contained only a single heavy chain Ig band (C\textsubscript{\gamma} probe) with germline configuration. This result indicates that the 18 kb and 14.5 kb fragments were of leukemic cell origin and could not have derived from genetic polymorphism.

Myeloid cell lines were also analyzed (Fig. 2). K562, HL-60, and KG-1 contained the heavy chain gene in the germline configuration, but ML-1 demonstrated rearranged DNA (12.5 kb) in the heavy chain region of the Ig gene (Fig. 2, lanes 2 and 3). Studies with this myeloblastic cell line, which has strong MPO and ANB activity and reacts with the myeloid-specific monoclonal antibodies anti-My-1 and SJ-D1, provided in vitro corroboration of our in vivo findings.

Comparison of the clinical and laboratory features of patients 1 and 2 with those of the other 12 patients revealed no striking differences, except that both patients were less than 2 yr old. Neither patient's leukemia cells contained the Philadelphia chromosome. The results of cytochemical staining are shown in detail in Table 2. Results of the TdT assay were negative for both patients.

All 14 children received daunorubicin and cytosine

<table>
<thead>
<tr>
<th>Stain</th>
<th>Percent Cells Positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodic acid-Schiff</td>
<td>25†</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>96</td>
</tr>
<tr>
<td>Alpha naphthyl butyrate esterase</td>
<td>73  22</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>98</td>
</tr>
</tbody>
</table>

* Differential counts for 100 cells.
† Fine granular staining.
arabinoside as induction therapy for ANLL. Patients 1 and 2 achieved complete remission, but the former died while undergoing a bone marrow transplantation; the latter remains in complete remission for 8+ mo on maintenance chemotherapy.

**DISCUSSION**

We present evidence for rearrangement in the heavy chain Ig gene of leukemic cell DNA from two patients with ANLL and one human myeloid cell line. Initially, we used a Cα-specific probe with *Bam*H1 enzyme-digested DNA. The results were confirmed with independently obtained samples that were digested with *Hind*III and run on separate gels. The specificity of the probe was confirmed with a J-region probe.

Because each of the patients studied had otherwise typical ANLL, heavy chain Ig rearrangement was unexpected, and phenotypic analysis was limited to myeloid-associated antigens. It will be important to test such patients for B cell-associated antigens and especially for cytoplasmic Ig, which could provide valuable insight into the functional capacity of the rearranged gene. However, when the ML-1 cell line was examined for lymphoid antigens (HLA-DR, CALLA, gp20, gp24, T11, T101, and the OKT series), none was found. This line was also TdT negative and did not possess cytoplasmic or surface immunoglobulins, suggesting that the rearranged heavy chain gene was nonproductive.

Our findings might distinguish a small clinically relevant subgroup of patients with mixed myeloid phenotypic characteristics and lymphoid genotypic features. This interpretation is consistent with previous demonstrations of mixed myeloid-lymphoid phenotypes in several patients with acute leukemia.3,4,33 Although both children entered complete remission after standard therapy for ANLL, the prognostic implications of their leukemias are not clear and will require additional study.

It now appears, from evidence in mouse non-B cells14,15 and human T leukemia-derived cell lines,2,16 that heavy chain Ig gene rearrangement is not restricted to cells of B lineage. Such rearrangement may also be present in cells of patients with T cell ALL (U. Rovigatti et al., manuscript in preparation). By contrast, light chain gene rearrangement and expression of functional heavy chains have been found only in B lymphoid cells.14,15 A plausible pathogenetic event in our two patients and the ML-1 cell line would be leukemic transformation in a progenitor cell capable of both B cell and myeloid differentiation. (A common lympho-myeloid progenitor cell has been proposed before, on the basis of lymphoid and myeloid blast crises in Philadelphia chromosome-positive chronic myeloid leukemia.34) Heavy chain Ig gene rearrangement in the progenitor cell would have to occur at an earlier stage of hematopoietic development than B cell commitment and would not be the stimulus for B-lineage development. This explanation is supported by clinical evidence suggesting that neoplastic transformation can affect progenitor cells capable of differentiation to more than one lineage.35 Alternatively, the genetic complement of some myeloid leukemia cells may not parallel that of normal cells, as predicted by models of lineage infidelity.36,37

Finally, it should be considered that the DNA rearrangements may not involve the Cα and J regions of the Ig gene. The 17-kb DNA fragment of the *Bam*H1 digest consists of multiple coding and noncoding sequences, and not all sequences are related to the Ig gene. It is possible that a rearrangement outside of the DNA sequences coding for the Ig gene is responsible for the change in fragment size we have identified. New probes hybridizing with DNA regions flanking the immunoglobulin gene will be required to test this hypothesis.

We conclude that heavy chain Ig gene rearrangement, although consistently found in ALL of B cell lineage, may also be present in some patients with otherwise typical ANLL. Future studies of leukemic cells should include analysis of light chain gene rearrangements, heavy and light chain mRNA expression, and immunoglobulin synthesis.

**ACKNOWLEDGMENT**

We wish to thank Drs. T. Rabbitts and C. Croce for their kind gift of Ig gene probes; Drs. C. Civin and J. Minowada for their gifts of monoclonal antibodies and cell lines; and Dr. D. Golde for the KG-1 cell line. We thank Drs. F. Bollum for the TdT antibody, M. Coleman for quantitative TdT assay, D. Williams for cytogenetic studies; and we thank J. Gilbert for editorial assistance.

**REFERENCES**


9. Max EE, Seidman JG, Leder P. Sequence of five potential recombination sites encoded close to an immunoglobulin K constant region gene. Proc Natl Acad Sci USA 76:3450, 1979
Heavy chain immunoglobulin gene rearrangement in acute nonlymphocytic leukemia

U Rovigatti, J Mirro, G Kitchingman, G Dahl, J Ochs, S Murphy and S Stass