Minimal Residual Disease in Hairy Cell Leukemia Patients Assessed by Clone-Specific Polymerase Chain Reaction

Evgeny Arons, Inger Margulies, Lynn Sorbara, Mark Raffeld, Maryalice Stetler-Stevenson, Ira Pastan, and Robert J. Kreitman

Abstract

Cladribine induces long-term complete remission in hairy cell leukemia (HCL) patients but does not clear minimal residual disease (MRD) according to high-sensitivity PCR assays. To quantify MRD in patients after anti-CD22 recombinant immunotoxin BL22 and other agents, we used a relative quantitative PCR (RQ-PCR) assay using a primer and probe, both patient-specific for the immunoglobulin heavy chain rearrangement. Using this method, we were able to detect one Bonna 12 HCL cell in either $10^6$ Jurkat cells or in $10^5$ normal mononuclear cells. We studied 84 samples from 10 patients, taken before or after treatment with BL22 and other agents. Patient-specific RQ-PCR was much more sensitive than flow cytometry, which in turn was (as recently reported) more sensitive than PCR using consensus primers. RQ-PCR was positive in 62 of 62 (100%) flow-positive samples in 10 patients and in 20 of 22 (91%) flow-negative samples in six patients. The relative level of MRD as quantified by RQ-PCR correlated with disease status and remission. Thus, patient-specific RQ-PCR is the most sensitive test for MRD in HCL patients and could be used to determine maximal response in patients obtaining multiple cycles of nonmyelotoxic biological treatment for this disease.

Hairy cell leukemia (HCL) is a clonal chronic B-cell lymphoproliferative malignancy, representing 2% of all leukemias, in which cells displaying CD19, CD20, CD22, surface immunoglobulin, CD79A, CD11c, CD40, and CD103 invade blood, bone marrow, spleen, and lymph nodes (1–3). The majority of patients require treatment for life-threatening pancytopenia or symptomatic splenomegaly. The most effective treatments, cladribine and pentostatin, can induce long-term complete remission (CR) in the majority of patients lasting a median of >5 years, and cladribine, which can be administered as a complete course in a single week, is most commonly used (4–6).

Despite the high CR rate and durability of response, therapy for HCL does not seem to be curative. A plateau on the disease-free survival curve has not been observed after many years of patient follow-up after purine analogues, indicating that patients eventually relapse (5). Fillouel et al. in 1994 reported Southern blots using patient-specific (clonogenic) probes obtained from surface immunoglobulin heavy (IgH) junction regions by PCR using consensus primers. By this method, evidence of circulating malignant cells persisted in seven of seven patients in long-term CR (7). Carbone et al. showed that whereas five of five HCL patients in CR had a negative Southern blot for a monoclonal rearrangement, all patients were positive in the first PCR step using consensus primers (8). If minimal residual disease (MRD) can be detected in the bone marrow biopsy by immunohistochemistry, HCL patients have a statistically significantly higher risk of relapse (9–12). Although HCL patients can be returned to CR with additional purine analogue therapy, the chance and length of expected CR are reduced (13), and long-term toxicity to T cells can result (14, 15).

With the advent of new biological therapies for HCL with mechanisms of action different from those of purine analogues, the ability to clear MRD again needs investigation. Rituximab is an unlabeled anti-CD20 monoclonal antibody, which kills cells via induction of apoptosis, complement-dependent cytotoxicity, and antibody-dependent cytotoxicity (16). Small clinical trials have reported a total of 18 (30%) CRs of 60 HCL patients (17–20). Of eight HCL patients in CR after rituximab, five proved negative by PCR using consensus primers, a technique sensitive until one IgH-positive cell was diluted into $10^5$ normal cells (21). BL22 is a recombinant immunotoxin containing truncated _Pseudomonas_ exotoxin fused to the variable domains of an anti-CD22 monoclonal antibody, which induces CRs in 19 (61%) out of 31 purine analogue-resistant HCL patients (22, 23). We have recently published that flow cytometry quantitation of MRD is more sensitive than PCR using consensus primers for FR3 IgH regions (24), and that most patients achieving CR to BL22 have no MRD by either PCR or flow cytometry (22, 23). To assess for the presence of MRD using a higher sensitivity test than flow cytometry, we did relative quantitative PCR (RQ-PCR) using sequence-specific...
probes and primers. MRD by this highly sensitive and specific test was compared with that assessed by methods previously published for HCL.

Materials and Methods

Cell lines. Jurkat and Bonna 12 cells were maintained in RPMI 1640 (Biosource, Camarillo, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) at 37°C in a humidified 5% CO2 atmosphere.

Patients and controls. Blood collected in heparin and EDTA tubes was obtained as part of sample acquisition protocols with informed consent approved by the National Cancer Institute Investigator's Review Board. Complete peripheral blood mononuclear cell immuno-phenotypic analysis was done as part of their standard evaluation. Flow cytometry was done as described (24). The diagnosis of HCL by flow cytometry also required the demonstration of monoclonality based on λ or κ surface light chains. For each patient, dates of blood collection for flow cytometry and PCR results were ≤5 days apart, except for one case in which for BH01 the RQ-PCR was done on day 29 of cycle 7 and the flow cytometry on day 21 of cycle 7. Peripheral blood specimens from five normal age-matched donors were also collected in EDTA tubes.

PCR studies of IgH gene rearrangement. Citrate-anticoagulated whole blood was centrifuged for 5 minutes at 1,800 rpm. The plasma layer was removed and replaced with PBS without Ca2+ or Mg2+. The blood-PBS mixture was layered over Ficoll (Biowhittaker, Walkersville, MD), and mononuclear cells were isolated, washed, pelleted, and frozen in dry ice. DNA was isolated from frozen pellets using the Nucleon BACC3 kit (Amersham Pharma Biotech, Piscataway, NJ) according to the manufacturer's instructions. Purified DNA was quantified by absorbance at 260 nm.

To determine clonality of the IgH gene, PCR was done on each sample using the IGH-Jα and VH5 framework 3 consensus primers and conditions as reported (25, 26). Briefly, 1 μg of DNA template was mixed with one PCR buffer, containing a 2.5 mM mL concentration of magnesium chloride, a 0.2 mM/mL concentration of deoxynucleotide triphosphates, a 0.5 μM/mL concentration of each primer, and LD ampli-Taq (Perkin-Elmer, Miami, FL) previously treated with Taq star antibody (Clontech, Palo Alto, CA). the DNA was amplified for 35 cycles (94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, with a final extension of 10 minutes at 72°C). After amplification, the products were separated by 16% nondenaturing PAGE (Bio-Rad Laboratories, Hercules, CA) and visualized by UV light after ethidium bromide staining. The expected molecular weight of the appropriate PCR products ranged from 70 to 120 bp. The PCR products were separated by 16% nondenaturing PAGE (Bio-Rad Laboratories, Hercules, CA) and visualized by UV light after ethidium bromide staining.

RNA extraction and cDNA synthesis. Total RNA was extracted from peripheral WBC using the QIamp RNA blood mini kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's instruction. The 25-μL reaction mixture containing 1 to 3 μg total RNA, 2 μL of 10 mM mL concentration of magnesium chloride, a 0.2 mM mL concentration of deoxynucleotide triphosphates, a 0.5 μM/mL concentration of each primer, and LD ampli-Taq (Perkin-Elmer, Miami, FL) previously treated with Taq star antibody (Clontech, Palo Alto, CA) the DNA was amplified for 35 cycles (94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, with a final extension of 10 minutes at 72°C). After amplification, the products were separated by 16% nondenaturing PAGE (Bio-Rad Laboratories, Hercules, CA) and visualized by UV light after ethidium bromide treatment. The expected molecular weight of the appropriate PCR products ranged from 70 to 120 bp. The PCR products were separated by 16% nondenaturing PAGE (Bio-Rad Laboratories, Hercules, CA) and visualized by UV light after ethidium bromide staining.

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Results

Patient characteristics. Peripheral blood specimens from 10 patients with a confirmed diagnosis of HCL were examined. All had clinical presentation and morphology consistent with HCL.

Flow cytometry in each case showed CD103 positivity and strong expression of CD22, CD20, and CD11c. HCL cells from all patients expressed κ or λ light chains. Patient BL14 had HCL variant (CD25 negative), and the other patients had classic HCL (CD25 positive). As shown in Table 2, all patients were men ages 45 to 74 years (median, 59 years). All patients were treated with BL22. Patients BL14 and BL41 received BL22 on a phase I trial; patients BL47 and BL49 received BL22 by special exemption; and BH01, BH02, BH04, BH05, BH07, and BH10 were treated on a phase II trial. Patients were at time points ranging from before to up to 64 months after enrollment and up to 20 months after the last cycle of BL22. Patients had received 1 to 31 cycles (median, 6 cycles) of BL22. Five of 10 patients had prior splenectomy.

Development of patient-specific RQ-PCR assay. To detect MRD using the standard PCR test for monoclonal B cells (HCL), DNA was prepared from citrated blood and subjected to PCR using IGH-Jα and VH5 framework 1 consensus primers (25, 26). The resulting band, containing the sequence of the surface IgH junctional region, was isolated from a gel, cloned, and sequenced, and specific primers and probes were designed. The types of IgH gene rearrangements found for the patients and the resulting primer sequences are listed in Table 1. Five patients were observed to have IGHJ4*02, two had IGHD1-26*01, two had IGHD6*02, and two had IGHV3-23*01. To detect HCL using sequence-specific PCR, total RNA harvested...
from patient blood in EDTA was used to produce cDNA. Quantitative PCR on the cDNA template was then accomplished using a consensus PCR primer combined with a specific primer and labeled specific probe.

Estimation of the sensitivity of patient-specific RQ-PCR. To define the sensitivity of the patient-specific RQ-PCR method, dilution tests were done, mixing the HCL cell line Bonna 12 with either the IgH-negative cell line Jurkat or mononuclear cells derived from equivalent mixture of five different healthy donors. PRKG1 gene expression detected by the PRKG1-specific primers/probe combination (29) was used for relative quantitation of Bonna 12–specific IgH expression (Fig. 1). For RQ-PCR amplification of the Bonna 12 IgH-specific fragment, we developed a Bonna 12–specific VH forward primer, because none of the VHFR2 consensus primers worked well due to high somatic mutation frequency in the VH region (Fig. 1). The lowest dilution of Bonna 12 cells that gave a specific signal was one Bonna 12 cell in either 10^6 Jurkat cells or 10^6 peripheral blood mononuclear cells from normal donors. Triplicates of cell dilution experiments for each cell dilution showed a coefficient of variance of <5%. (Fig. 2).

Detection of HCL by flow cytometry and PCR. Of the three tests used for measurement of HCL in blood, flow cytometry, consensus IgH-PCR, and patient-specific RQ-PCR, the most sensitive was patient-specific RQ-PCR, with 82 of 84 (98%) samples found positive. Flow cytometry was less sensitive, with 62 of 84 (74%) samples found positive. Consensus IgH-PCR was least sensitive, with 32 of 58 (55%) samples found positive.

Comparison of RQ-PCR and consensus IgH-PCR. Of 58 samples from the 10 patients tested in parallel by patient-specific RQ-PCR and consensus IgH-PCR, consensus IgH-PCR revealed a clonal band pattern in 32 samples, a polyclonal distribution in 23 samples, and no signal in three samples. Of the 10 patients, samples from eight patients initially contained clonal bands by IgH-PCR, and this test became negative in three (BH02, BH05, and BL49). Of the 58 samples tested by consensus IgH-PCR, 56 (97%) were positive by patient-specific RQ-PCR. This includes 32 of 32 (100%) samples with monoclonal and 21 of 23 (91%) samples with polyclonal pattern by consensus IgH-PCR, in addition to 3 of 3 (100%) samples with indeterminate consensus IgH-PCR. The three samples with neither polyclonal nor monoclonal consensus IgH-PCR showed a low WBC count.

Table 1. Primers and probes for RQ-PCR

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgH gene rearrangement</th>
<th>Junction region</th>
<th>Sequences of patient-specific primers and probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL14</td>
<td>IGHV4-31<em>04 IGHD6-13</em>01 IGHJ4*02</td>
<td>TGTGCCAGAGAGAGCGCAG-CTGGCACATATTCTTGGACGCTG</td>
<td>TM, CTCGGCTCCCTTCTTGGACGCTGAC</td>
</tr>
<tr>
<td>BL41</td>
<td>IGHV1-2<em>03 IGHD3-3</em>01 IGHJ1*01</td>
<td>TGTGCCAGAGAGGCCCGGATTCTATTCTTGGACGCTG-</td>
<td>PR, CCAAGTGACAAATATAAGTGGCC</td>
</tr>
<tr>
<td>BL47</td>
<td>IGHV3-7<em>01 IGHD4-23</em>01 IGHJ2*01</td>
<td>TTATGTTGTTGTTAACAGGCTTCTG</td>
<td>TM, AGAAGGCTATTACTATGGATAGT</td>
</tr>
<tr>
<td>BL49</td>
<td>IGHV1-2<em>02 IGHD3-22</em>01 IGHJ6*02</td>
<td>TTATGTTGTTGTTAACAGGCTTCTG</td>
<td>PR, AGACGTCCAAGCAGGAAGG</td>
</tr>
<tr>
<td>BH01</td>
<td>IGHV3-23<em>01 IGHD3-9</em>01 IGHJ3*02</td>
<td>TTATGTTGTTGTTAACAGGCTTCTG</td>
<td>TM, CCAAAACAGCTAATAGGAC</td>
</tr>
<tr>
<td>BH02</td>
<td>IGHV1-3<em>01 IGHD1-26</em>01 IGHJ4*02</td>
<td>TTATGTTGTTGTTAACAGGCTTCTG</td>
<td>PR, CAGATTCCAAGGCAGGGC</td>
</tr>
<tr>
<td>BH04</td>
<td>IGHV3-11<em>03 IGHD2-2</em>01 IGHJ4*02</td>
<td>TTATGTTGTTGTTAACAGGCTTCTG</td>
<td>TM, ACTCCGCCTCCGACCATACACA</td>
</tr>
<tr>
<td>BH05</td>
<td>IGHV3-30<em>10 IGHD1-26</em>01 IGHJ4*02</td>
<td>TTATGTTGTTGTTAACAGGCTTCTG</td>
<td>PR, CCAAGTGACAAATATAAGTGGCC</td>
</tr>
<tr>
<td>BH07</td>
<td>IGHV6-1<em>02 IGHD3-10</em>01 IGHJ6*02</td>
<td>TTATGTTGTTGTTAACAGGCTTCTG</td>
<td>TM, AGAAGGCTATTACTATGGATAGT</td>
</tr>
<tr>
<td>BH10</td>
<td>IGHV3-23<em>01 IGHD2-8</em>01 IGHJ4*02</td>
<td>TTATGTTGTTGTTAACAGGCTTCTG</td>
<td>PR, CCAAGTGACAAATATAAGTGGCC</td>
</tr>
</tbody>
</table>

Abbreviations: TM, Taqman probe; PR, sequence-specific primer.

*JH consensus primer.
(2.38, 2.39, and 3.00) and low percentage of B cells (1%, 8.5%, and 0.28%). Thus, patient-specific RQ-PCR was more sensitive than consensus IgH-PCR; the former was always positive when the latter was monoclonal and usually positive when the latter was polyclonal.

**Comparison of patient-specific RQ-PCR and MRD flow cytometry.** In all 84 samples from 10 patients, we simultaneously analyzed circulating HCL cells using flow cytometry and patient-specific RQ-PCR (Figs. 3-4). We found that RQ-PCR was positive in 62 of 62 (100%) flow-positive samples and in 20 of 22 (91%) flow-negative samples. Thus, flow cytometry was more sensitive for HCL than was consensus IgG-PCR, and patient-specific RQ-PCR was more sensitive than either of these two tests.

**HCL disease burden analysis over time and in relation to treatment.** To quantify MRD by patient-specific RQ-PCR, expression was determined as a percentage of PRKG1 housekeeping gene expression by RQ-PCR. As an negative control, we used a cDNA mixture of peripheral blood mononuclear cells from five normal donors (27). Results were considered positive only when there was a detectable signal in the sample and no reaction in normal cDNA. Patient-specific RQ-PCR analyses were done in triplicate, and the level of patient tumor cell IgH expression was determined. The results are shown in Fig. 3 for phase II patients and in Fig. 4 for the earlier phase I and special exemption patients. The sensitivity of detection in each patient was expected to be different because the patient-specific primer/probe combinations were unique. In addition, the concentrations of malignant cells in the blood by flow cytometry, expressed as % HCL cells divided by the WBC, were shown on the same figures.

**HCL disease burden in patients receiving BL22 on phase II.** To determine the effect of BL22 on HCL disease burden by PCR and flow cytometry, we tested patients on phase II, because in these more recently treated patients, IgH sequences could be cloned and patients tested before initiating BL22. As shown in Fig. 3, patients BH01, BH02, BH05, and BH07 had more than a 1-log decrease in disease from before BL22 to 1 month after beginning the first cycle by both RQ-PCR and flow cytometry. During the first BL22 cycle, the RQ-PCR relative IGH expression level decreased from 3.1 ± 0.006 to 0.029 ± 0.016 for BH01, from 231 ± 3.796 to 0.210 for BH02, from 1,523 ± 140 to 26 ± 4 for BH05, and from 0.104 ± 0.016 to 0.0027 ± 0.0006 for BH07. Patient BH10 responded more slowly but eventually had decreases in disease by both tests. Disease by flow cytometry became consistently undetectable in patients BH01 and BH02 by 2.5 and 3.5 months after initiating BL22 and was undetectable twice in BH05 at 10 and 12.5 months after initiating BL22. Relative expression in BH05 decreased from 1,523 to a minimum of 0.022 after 10 cycles and by 11 months after beginning BL22, a change of nearly 5 logs. Figure 3 shows that decreased disease burden correlated with negative bone marrow biopsies in patients BH01, BH02, BH05, and BH07. Bone marrow biopsies in these patients normalized after cycles 1, 2, 10, and 1 of BL22, respectively, at time points that were 1.5, 3.5, 11.5, and 4.5 months after initiating BL22. After cycle 10 of BL22, patient BH05 had MRD in the blood by flow cytometry on one of three time points, but the bone

![Fig. 1.](sequence_of_bonna_12_igh_vf1r1_jj_fragment_and_location_of_bonna_12_specific_igh_rearrangement_probe_and_primers.jpg)
marrow biopsy immunohistochemistry showed a CR with no MRD on the last two assessments. For patient BH04 (Fig. 3C), at the final assessment, the circulating HCL cells increased by flow cytometry, but the RQ-PCR decreased. The patient had an infection at that time. RQ-PCR was undetectable in only two samples, the last two assessed for patient BH01 (Fig. 3A). Other tests of MRD were also negative at those time points for that patient. Thus, patient-specific RQ-PCR correlated with response in HCL patients treated with BL22 and was more sensitive than other measures of residual HCL.

**HCL disease burden in patients after BL22 and other therapeutic agents.** To investigate patient-specific RQ-PCR in patients receiving chemotherapy or other agents for HCL, we tested HCL patients who were previously enrolled on the phase I trial (BL14 and BL41) or who were enrolled by special exemption before phase II testing (BL47 and BL49). Patient BL14 (Fig. 4A), who previously had CR to BL22, had slowly decreasing disease burden during cycles 19 to 31 given several years after relapse. During this time, the disease by RQ-PCR decreased from 2,100 ± 580 to 4.5 ± 1.3, a change of 2.7 logs over nearly 14 months. Disease by flow cytometry during the same interval decreased 2.1 logs from 3,500 to 29 cells/μL.

Despite resistance to rituximab and pentostatin when administered separately before cycle 1 of BL22, this patient had CR to the rituximab/pentostatin combination 59 months after beginning BL22. MRD was undetectable in the blood by flow cytometry at this time.

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**Fig. 2.** Determination of RQ-PCR sensitivity for detection of minimal Bonna 12 cell number in serial dilutions of known amounts of cells. A, with IgH-negative Jurkat cell line. B, in normal peripheral blood mononuclear cells (PBMC) from five different donors.

**Fig. 3.** Level of circulating HCL cells, phase II. At the indicated points in time after initiation of the BL22 phase II protocol, the log of relative expression by sequence-specific quantitative PCR (●) and % HCL cells by flow cytometry relative to total WBC (▲). The highest horizontal line indicates undetectable results (Neg). Bone marrow biopsy (BM) results listed include disease present (D), consistent with CR with MRD by immunohistochemistry (M) and CR without MRD (N). The row for treatment (RX) includes the timing of cycles of BL22 (▲).
cytometry but could be quantified by RQ-PCR at $1.3 \times 10^{-5}$ $\pm$ 3.4 $\times 10^{-7}$. As shown in Fig. 4B, patient BL41, who had partial remission to BL22, had many samples where HCL could not be detected by flow cytometry, but HCL burden could be quantified at all time points by RQ-PCR. Patient BL47 had slowly decreasing disease burden by both RQ-PCR and flow cytometry (from 670 $\pm$ 75 to 390 $\pm$ 59 and from 15,000/µL to 3200/µL) followed by more rapid response to combination rituximab and granulocyte macrophage colony-stimulating factor (to 42 $\pm$ 4.6 and 75/µL), and throughout the two curves were somewhat parallel (Fig. 4C). Finally, patient BL49, who had prior partial remissions to cladribine, had a partial remission to BL22 followed by a transient CR to pentostatin. Flow cytometry became undetectable once, but RQ-PCR remained detectable, albeit at a very low level 6 logs below baseline (30 $\pm$ 4.6 to 2.8 $\times$ 10$^{-5}$ $\pm$ 4.7 $\times$ 10$^{-5}$). Thus, decreases in HCL burden as assessed by RQ-PCR were observed not only after BL22 but also after rituximab and pentostatin.

Discussion

The goal of this study was to characterize a high sensitivity PCR assay for HCL because HCL eradication has not been achieved after cladribine and is unknown after newer agents, including BL22 anti-CD22 recombinant immunotoxin. A total of 84 samples from 10 HCL patients were tested using both flow cytometry and a new quantitative PCR assay (RQ-PCR) using patient-specific primer/probe combinations to surface IgG rearrangements. We confirmed that flow cytometry is more sensitive than PCR using consensus primers and showed that patient-specific RQ-PCR was much more sensitive than either test.

Analysis of MRD in HCL has focused on immunohistochemistry of the bone marrow or flow cytometry of blood (10–12, 31–34), and only a few studies have used PCR. As mentioned above, PCR using consensus primers followed by Southern blotting using patient-specific probes identified HCL in all patients in CR after 2-chlorodeoxyadenosine (7, 8). This method was sensitive for HCL after dilution of DNA 1:10$^4$ or 1:10$^5$ (7, 35). We reported that PCR of sequences encoding surface IgH using consensus primers was less sensitive than flow cytometry, the latter able to detect below one HCL cell in 10$^5$ normal cells (24). Capillary electrophoresis after fluorescent PCR using consensus primers allowed detection until one IgH$^+$ cell was diluted into 10$^5$ normal cells (21) and showed that MRD cleared in five of eight with CR, suggesting that rituximab might finally be an agent capable of eradicating HCL. We reasoned that assessing eradication of HCL would require even higher sensitivity PCR tests, previously reported for chronic lymphocytic leukemia and acute lymphoblastic leukemia. Fluorescent PCR of samples from precursor B-cell acute lymphoblastic leukemia resulted in detection sensitivity of 2 $\times$ 10$^{-6}$ (36). PCR of the CDR3 region using clonotypic primers followed by imaging on a Metaphor gel resulted in detection of one chronic lymphocytic leukemia cell in 10$^5$ polyclonal cells (37). In RQ-PCR of acute lymphoblastic leukemia, optimal sensitivity (down to 3.16 $\times$ 10$^{-6}$) could be obtained using either clone-specific probe and consensus (germline) primers, or consensus probe with clone-specific primer (38). In a recent comparison of clone-specific RQ-PCR with four-color flow cytometry, the former was found to be much more sensitive for detection of MRD in chronic lymphocytic leukemia, with detection sensitivity down to 10$^{-5}$ (39). Thus, our RQ-PCR assay, sensitive to 1 in 10$^5$ cells, compares well to detection of MRD in chronic lymphocytic leukemia and acute lymphoblastic leukemia, is the first demonstration of clone-specific RQ-PCR in HCL, and is the first comparison of flow cytometry and RQ-PCR for detection of MRD in this leukemia.

It should be mentioned that the sensitivity of the clone-specific RQ-PCR method might be different in different patient samples because the clone-specific primers and probes are different in each case. Nevertheless, RQ-PCR was never less sensitive than either quantitative four-color flow cytometry for HCL, or consensus IgH-PCR. In addition, quantitation relative...
to a standard housekeeping gene (PRKG1) does not allow an absolute quantitation of the number of target cells per microliter as does quantitative flow cytometry. In fact, it is possible, as previously proposed, that RQ-PCR could detect RNA fragments from apoptotic HCL cells, which escaped flow cytometry detection (40). Although RQ-PCR is more sensitive, it is also more labor intensive and costly. Thus, RQ-PCR in HCL may be particularly helpful when flow cytometry is no longer able to detect disease.

Of our 10 HCL patients studied, six achieved CR, and in only one (BH01) of these six patients was HCL undetectable by RQ-PCR. To determine whether HCL can be eradicated by BL22 or by other biological agents, many more patients with RQ-PCR negativity will need to be studied, each for many years. Nevertheless, it is interesting to note that this patient received six cycles of BL22 after the bone marrow became negative by flow cytometry and five cycles after the flow cytometry became negative. It was only after the last cycle of BL22 that RQ-PCR was resolved. It is possible that this patient had a collection of HCL cells outside the blood, which will cause relapse but which could have been eradicated by additional cycles of BL22 after becoming RQ-PCR negative.

Because HCL is an indolent disease, many years of follow-up after both 2-chlorodeoxyadenosine and/or BL22 will be required to determine the true clinical significance of RQ-PCR. Clearly, the most sensitive test previously reported, flow cytometry of the blood (24), is not adequate because many patients relapse after chemotherapy, rituximab, or BL22 renders patients negative by this test. It is possible that at some very low but detectable level of RQ-PCR positivity, patients will not relapse even after decades of follow-up. Conversely, it may be that patients with HCL burden even lower than 1 in 10<sup>6</sup> cells could eventually relapse. Our RQ-PCR assay could potentially be improved further, either by improving primer or probe sequence, or by improving detection systems. Improving detection of HCL was previously irrelevant clinically, because the cumulative toxicity of purine analogues prevented the administration of extra cycles to eliminate MRD. Because significant toxicity of BL22 has not been observed with the 4th or later cycles, even up to 31 cycles in HCL, it may be possible to keep treating MRD as long as it is decreasing, or until it is undetectable. Studies with RQ-PCR may be crucial to determining the number of cycles of BL22 for optimal response, and to determine if HCL can be, perhaps for the first time, eradicated in some patients.

### Acknowledgments

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