Long-Term Follow-up of Residual Disease in Acute Lymphoblastic Leukemia Patients in Complete Remission Using Clonogeneic IgH Probes and the Polymerase Chain Reaction

By Y. Nizet, S. Van Daele, P. Lewalle, J.L. Vaerman, M. Philippe, C. Vermylen, G. Cornu, A. Ferrant, J.L. Michaux, and P. Martiat

We sequentially studied bone marrow (BM) samples of 25 patients in complete remission of an acute lymphoblastic leukemia (ALL) using a simplified polymerase chain reaction (PCR) strategy (direct use of the PCR product as a clonogenic probe recognizing rearranged Ig heavy chain sequences) as a first approach. BM aspirates were serially investigated after obtention of a complete response. When sensitivity was less than 1:10^6, the PCR fragment was sequenced and a specific oligonucleotide was synthetized and used as a probe (five cases). Cases in which minimal residual disease (MRD) became undetectable were cross-controlled using either TCRβ rearrangement or a specific translocation to circumvent the problem of false-negative results arising from clonal evolution. The median follow-up was 30 months (3 to 61 months). Within the first 3 months of complete remission, MRD was detectable in 22 of 23 investigated patients and remained so in 19 of 21 patients examined at 6 months, regardless of the long-term clinical outcome. In patients remaining in complete remission at 30 months or more, two patterns of MRD emerged during the follow-up. Either it continuously decreased to ultimately become undetectable (five patients) or remained detectable (five patients) with an increase after discontinuation of treatment in two. In the eight patients who relapsed, MRD persisted throughout the clinical course, and eventually increased 3 to 12 months before relapse was clinically detectable. In one case, clonal evolution of the VDJ heavy chain region was observed and recurrence of MRD shown by the use of TCRβ rearrangement as a control. We conclude that the use of this simplified methodology is a valuable tool for the follow-up of MRD in a majority of ALL patients, though in a few cases, sequencing needs to be performed to achieve a relevant sensitivity. The possibility of clonal evolution requires a cross-control of any sample becoming negative whatever the initial rearrangement used to generate a probe. In patients on therapy, sequential search for MRD seems to be a good tool for predicting the long-term outcome. In addition, patients remaining positive at the time treatment is discontinued or with a high tumor burden after a few months therapy may be at a higher risk of subsequent relapse, although a longer follow-up is needed to answer this question.

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In the past few years, several investigators including ourselves have monitored residual leukemic cells (minimal residual disease or MRD) in acute lymphoblastic leukemias (ALL) in complete remission using the polymerase chain reaction (PCR) and clonogenic probes derived from the TCRγ-δ or the IgH-rearranged genes. From the published series available so far, some facts are emerging but remain to be confirmed on a larger scale. First, it seems that eradication of malignant cells is a process that always take several months, and that most patients investigated at short term after induction are positive until late in treatment. Therefore, a single positive result does not seem to be a good predictor of relapse for samples taken during the initial months of therapy. Second, for patients at the end or posttreatment, a negative result appears to predict long-term disease-free survival but this remains controversial. As a matter of fact, a recent report by Ito et al shows that PCR negativity at this stage of the clinical course is not sufficient for ruling out subsequent relapses. Third, persistence of a high residual disease at the end of induction therapy may also have a bad prognostic significance. Finally, in patients who eventually relapse, persistence or even increase of MRD throughout the course of treatment seems to be an important point.

Although these preliminary informations are of major importance, they need to be further clarified. Moreover, other problems have to be solved before this technology can lead to modulation of therapy according to variations of MRD. In particular, several authors have brought convincing evidence for clonal evolution (that can occur in both types of rearranged sequences, although more frequently in the IgH genes) during the course of the disease. Nevertheless, the incidence of clinically relevant clonal for a given PCR strategy remains poorly known. The optimal strategies to circumvent this problem are still to be better defined. Other practical questions such as the interval between an increase in MRD and the occurrence of relapse, or the relevant level of sensitivity, if any, that must be achieved have also to be more completely answered.

We tried to partially address these questions by following all the patients presenting in our institution since 1989 with a diagnostic of B-lineage ALL and a PCR-detectable rearrangement of the Ig heavy chain genes. MRD was monitored using the simplified PCR methodology described previously as a first approach. Every effort was made to simultaneously keep the methodology as simple as possible (by increasing its degree of complexity only when it appeared needed) and to achieve a high degree of reliability (by
using cross-controls each time we felt to be at risk of encountering false-negative results.

**PATIENTS AND METHODS**

**Patients.** Between January 1989 and March 1993, bone marrow (BM) samples of 25 patients with a diagnosis of B-lineage ALL were studied at diagnosis and during follow-up after they achieved complete remission according to standard hematologic and morphologic criteria. There were 20 children and 5 adults. The clinical and biologic data of these patients are summarized in Table 1. They were treated according to the FRALLE29 (for the children) and the LALA 8721 (for the adults) protocols, respectively.

The only prerequisites for inclusion in this study were the availability of initial and subsequent BM material, presence of a monoclonal IgH gene rearrangement shown by Southern blot analysis at diagnosis, and the possibility to detect this rearrangement using the PCR as described previously.7

**Southern blot.** High molecular weight DNA was prepared from mononuclear BM cells, digested using Eco RI and HindIII, electrophoresed through agarose gel, transferred to a nylon membrane (Zetaprobe; Biorad, UK) and hybridized to a 32P-labeled JH gene probe22 using standard DNA technology.22 Southern blot analysis showed the presence of a rearranged band in all 16/19 patients with a diagnosis of B-lineage ALL.

**Clonogenic probes.** The PCR and the direct use of the PCR product as a clonogeneic probe has been described previously7 except that high performance liquid chromatography (HPLC) was used instead of FPLC to recover the PCR products. The rearranged bands were purified on an ion exchange column (Gen-Pak Fax; Waters, Milford, USA). The chromatography conditions were as follows: buffer A (NaCl 0 M/Tris HCl pH 8.0 25 mmol/L), buffer B (NaCl 1 M/Tris HCl pH 8.0 25 mmol/L), gradient (35% to 65% B in 35 minutes). Briefly, 35 cycles of PCR were performed with an annealing temperature adjusted in each patient to optimize the yield of the procedure.

Two oligonucleotides recognizing V<sub>H</sub> and J<sub>H</sub> consensus sequences were used: 5'-CCAGAGGACACGGCGGTATTACTG-3' end of FR3 of V<sub>H</sub> genes; 5'-AUCTGCTGAGGACGGT-GACC-3' end of J<sub>H</sub> segments.

The rearranged band was then purified by HPLC (using the same chromatography conditions) and digested with the restriction enzyme Sau 96 I recognizing a site immediately 5' of the 3' amplimer Ca2 (Fig 1). The digestion products were separated using the same chromatography technique and the band containing the clonospecific sequences was isolated for direct use as a probe. The probe was labeled using a specific hexanucleotide, 5'-TACTGT-3', complementary to the 3' end of amplimer Ca1 to initiate the reaction (Fig 1). If more than one rearranged band were present at diagnosis, each was used to derive a probe. In these cases, the PCR products were blotted in the same way and separately hybridized to each of the probes. Dilutions of the initial material into peripheral blood (PB) mononucleated cells were used as a semiquantitative measurement of the number of residual cells. By comparison to our first report,7 two modifications were introduced to take into account the lack of sensitivity of certain PCR products when used directly as hybridization probes and the possibility of clonal evolution: (1) sensitivity was first assayed on dilutions of initial material. If it was inferior to 1:10<sup>4</sup>, sequencing was performed. The amplified products to be sequenced were obtained by performing the same PCR after replacement of one of the primers by its biotinylated analog.

**Table 1. Patients Characteristics at Diagnosis and Sensitivity of the Probes**

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<th>Patient</th>
<th>Age/Sex</th>
<th>Organomegaly</th>
<th>WBC count (10&lt;sup&gt;9&lt;/sup&gt;/L)</th>
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**Abbreviations:** WBC, white blood cell; NP, not possible; Direct, direct use of the PCR product as a clonogeneic probe; Oligo, probe obtained after sequencing; Control, technique used for the control of a negative result (TCRG rearrangement or specific translocation); Pro-B, CD19+; CD34+, CD10−; Pre-B, CD19+, CD10+, CY<sub>A</sub>−; Pre-pre-B, CD34+, CD19+, CD10+, CY<sub>A</sub>−; Early B, CD10+/−, CD20−, SmI<sub>G</sub>−, CD5−.
Single-stranded DNA was prepared with Dynabeads M-280 (Dynal, Oslo, Norway) and sequenced using the dideoxy chain termination method.\textsuperscript{24,25} Eighteen-mer oligonucleotide probes were synthesized using a Cyclone DNA synthetizer (Applied Biosystems, Foster City, CA) and were designed to span the D-N-J region to optimize the reaction for the detection of leukemic hybrid mRNAs generated by the translocations t(9;22) or t(1;19) rearrangement according to the method described by Hanssen-Hagge et al.\textsuperscript{2} if existing, or RT-PCR for the detection of leukemic cells in the follow-up BM samples. Eight patients have relapsed at the time of the analysis. In these patients, the level of MRD increased several months before the relapse was detectable by conventional ways. However, the interval between the increase in MRD and the clinical relapse was variable, ranging from 3 to 12 months. In patient no. 2 a BM aspiration, though not strictly meeting the criteria for relapse, showed the reappearance of a few cells (5%) bearing the same morphologic (L3 ALL) characteristics as the initial leukemic cells. None of the 7 patients having relapsed have shown evidence of clonal evolution. However, in one patient (no. 7, Fig 4), in which no clinical signs of disease are present so far, this phenomenon occurred, despite the fact that this patient was followed using a synthetic oligonucleotide probe spanning the D-N-J region, which theoretically should lessen the risk of missing a clonal evolution.\textsuperscript{18,19} In this case, TCRδ rearrangement was used to generate a probe when MRD became undetectable (30 months) using the IgH probe (Fig 5). Further follow-up of the patient showed a recent increase of his MRD, detectable using the TCRδ probe, with the appearance of two shorter VDJ\textsubscript{H} rearranged bands detected on the ethidium bromide gel at the last PCR. These new bands did not hybridize to the initial VDJ\textsubscript{H} clonogenic probe (Fig 5).

In the seven patients remaining in complete remission at the time of the analysis and in whom MRD ultimately became undetectable, residual leukemic cells could be detected up to 24 months after start of therapy, but progressively decreased to eventually disappear. The time at which MRD became undetectable in these patients was quite variable, ranging from 1.5 to 30 months and did not correlate with any clinical or biologic feature at diagnosis. In these 7 patients and three bands in one. In these four patients, all rearranged bands were used as probes to look for residual leukemic cells in the follow-up BM samples.

\textbf{Sensitivity.} Using the initial PCR product as a probe, the limit of sensitivity varied from $1:10^5$ to $1:10^3$ using the dilution of the initial samples as a semiquantitative measurement. In 5 cases, the limit was $1:10^3$. It was $1:10^4$ in 8 and $1:10^3$ in 12 patients. One patient (no. 12, Fig 3) who eventually relapsed 13 months after the obtention of the first CR, was initially followed using the simplified strategy. The sensitivity obtained with this probe was $1:10^3$. Using this probe, we were not able to detect any increase of MRD before the relapse became clinically obvious. At this moment (1991), an oligonucleotide probe was synthetized achieving a detection threshold of $1:10^3$. The use of this probe allowed the detection of a continuous increase in the amount of residual leukemic cells 12 months before reappearance of the disease.

\textbf{Clinical results.} MRD was detectable, regardless of the long-term outcome in 24 of 25 patients investigated after completion of the induction (1 month), 22 of 23 patients at 3.0 months, and 19 of 21 patients at 6.0 months. The data obtained during follow-up are summarized in Fig 4 (patients still in complete remission [CR] at the time of the analysis) and Fig 3 (patients having clinically relapsed). Except in one patient (no. 22, Fig 4), all the BM samples tested within the first 3 months after induction of complete remission were found positive.

\textbf{Patients.} Out of 33 ALL patients in whom a rearranged band could be detected at diagnosis by Southern blot techniques using a J\textsubscript{μ} probe, 28 (84\%) had a rearrangement detectable using PCR. In 25 out of these 28, subsequent BM samples were available for analysis. A double rearrangement with deletion of the germ-line band was seen in three
patients, 6 could be cross-controled using TCRβ rearrangement (four cases) or RT-PCR detecting a specific translocation: t(9;22) and t(1;19). These controls confirmed the negativity of MRD.

Twelve patients treated with conventional chemotherapy could be investigated at 24 months at the time treatment was discontinued according to the protocol. Five of them (nos. 1, 5, 7, 8, and 9, Figs 3 and 4) were still positive. Two of them (nos. 5 and 8, Fig 3) have relapsed 12 and 30 months after cessation of treatment. In the three other patients (nos. 1, 7, and 9), the follow-up is too short (3, 6, and 6 months) to draw any conclusions yet.

Interestingly, in two patients undergoing autologous BM transplantation with unpurged positive (1:10⁴) marrow, MRD decreased after a few months and ultimately became undetectable. These two patients are still in CR, 19 and 12 months after the procedure.

**DISCUSSION**

In our previous preliminary study, we had shown that, in 80% of the B-lineage ALL, a follow-up of residual disease using a simplified methodology for obtaining clonospecific probes derived from the VDJμ rearranged sequences of the Ig heavy chain genes, was achievable with a sensitivity of 1:10⁴ to 1:10⁵. With a larger group of patients, we consider that the sensitivity is too low in approximately 20% of the cases, which implies that a sequencing step has to be performed to synthesize an oligonucleotide probe. Having missed more than 10 months of continuous increase of MRD, in a patient followed using a probe with a sensitivity of 1:10³, has prompted us to adapt our strategy in 1991, and to synthesize oligonucleotide probes for approximately 20% of our patients. Clearly, methods achieving a level of sensitivity of 1:10⁴ may lose most of the advantages offered by the early detection of an unfavorable course, as shown by the case reported here. Therefore, we think that a sensitivity of at least 1:10⁴ cells is necessary to avoid gross underestimation of MRD. On the other hand, the best one can achieve, given the amount of DNA used in a PCR reaction (1 to 2 μg), is probably not much better than 1:10⁵. We have at least one patient who was found transiently negative, with this level of sensitivity, before detectable MRD showed up.
again. This shows the limitations of PCR monitoring of MRD, even with a very good sensitivity. PCR will never be able to tell what is going on below the level of 1 malignant cell diluted in $1 \times 10^6$ normal ones. In other terms, PCR negativity may well be more relevant than negativity obtained with classical methods, but is in no way equivalent to readication of the disease. Those patients who become negative not only to be cross-controlled to avoid the problem generated by clonal evolution, but also to be repeatedly investigated afterward, especially if treatment is discontinued.

Two other issues raised by the use of IgH rearrangement-based strategies are the presence at diagnosis of more than one clone or the emergence during the course of the disease of a modified IgH gene rearrangement. The problem of different subclones at diagnosis can be dealt with rather easily because a Southern and a PCR analysis are performed on initial material. If more than one rearranged band is present, a probe can be derived in the same way from each of the rearrangements, and all are separately used to screen the PCR products during the follow-up. Not all the probes in this case have the same sensitivity, but we consider a result as positive as long as one of the probes detects residual cells.

Clonal evolution during follow-up is more concerning and proper precautions have to be taken to avoid misinterpretations of negative results. This event is reported to occur frequently, but according to Rovera et al., a clonal evolution detected at the Southern level might result in a change in the crucial sequences around the diversity region in only a smaller proportion of the cases. The percentage of cases in which it really occurs may be a controversial subject, and strategies aiming at rendering it less likely to interfere with the recognition of leukemic cells by the probe are interesting but do not provide a totally satisfactory answer. The only patient in our series in whom that problem happened was precisely followed with an oligonucleotide probed covering the D-N-J portion of the VDJ rearranged sequences. If we chose not to perform a cross-control, we should then accept to miss a recurrence of MRD indicating a relapse in 5% to 10% of our patients, even using oligonucleotide probes. The fact that this might happen, even in a small number of patients, would make PCR unreliable to adapt the treatment in a particular patient. The method consisting of screening patients at diagnosis for the presence of PCR detectable rearrangements of TCRγ and Ig heavy chain genes and to cross-control any negative result makes the PCR follow-up a little more complex, but the gain in reliability seems worthwhile to us. Indeed, if the follow-up of MRD is to translate into modulation of therapy, we feel that we may not afford to provide possibly wrong information that would lead to a lower intensity of therapy in some patients who would rather deserve treatment intensification. In our mind, this can make the difference between a statistically relevant methodology and a technique allowing individually adapted treatment strategies.

As far as clinical relevance of detection of MRD in ALL is concerned, our data show that for patients on treatment (within the first 2 years of diagnosis), its behavior in semi-
quantitative terms rather than its persistence at a particular moment, appears to be related to the outcome of therapy. The patients who seem to have a favorable long-term outcome show a decline in the level of detectable MRD along the course of the treatment, while in the patient who eventually relapse, this level had a tendency to remain stable or to progressively increase. This conclusion is in rather good agreement with the data published by Yokota et al. about a series of 27 patients using a strategy based on the detection of TCRα sequences and with our preliminary results. The previous observations showing that eradication of malignant cells is a process that always take several months, and that most patients investigated at short term after induction are positive until late in treatment have been confirmed. Therefore, a single-positive result is not a good predictor of relapse for samples taken during the initial months of therapy. In patients who will eventually relapse, persistence or even increase of MRD throughout the course of treatment seems to be the important point. As suggested previously, for patients at the end or posttreatment, a negative results renders more likely a long-term disease free survival. Nevertheless, PCR negativity has to be regarded as a relative concept (nothing being detected), more relevant than classical complete remission, but in no way equivalent to disease eradication. A recent study reported by Ito et al. provides evidence that PCR negativity at the end of treatment may not be sufficient to assure the cure and that longer serial follow-up is required. In this study, most of the patients were found negative at this stage of follow-up. The level of sensitivity was one malignant lymphocyte in an average of $2.2 \times 10^4$ B-cells. Our findings are slightly different because we found 5 out of 12 patients still positive at the end of treatment, using probes the sensitivity of which (estimated by dilutions into PB mononucleated cells DNA) was $1:10^4$ for one and $1:10^5$ for the other four patients. Whether this difference is a matter of sensitivity (although this looks unlikely) or reflects heterogeneity in the patients who are compared is difficult to tell from such small series. Anyway, our results do not prove that patients found negative at that stage of the clinical course are definitively cured, but rather suggest that persistence of detectable MRD when therapy is discontinued has bad prognostic significance. But the follow-up of patients with such a finding is still too short, and their number too small, to draw definitive conclusions. The same authors found the persistence of a high residual disease at the end of induction therapy to be significantly correlated with long-term prognosis. This was not observed in our series but again the number of our cases is too small to examine this point in a statistically significant way. In conclusion we feel that, provided adequate modification are brought to strategies designed in the years 1989 to 1990, to overcome two major problems (clonal evolution and lack of sensitivity in some cases), the use of PCR with clonogenic probes derived from the IgH-rearranged sequences is a valuable tool that, if the results are confirmed, will most probably help to adjust individual treatment in the future.
A. PCR OF THE IgH REGION IN PATIENT NR 5

ETHIDIUM BROMIDE STAINED GEL

HYBRIDIZATION WITH THE D-J OLIGONUCLEOTIDE PROBE

B. PCR OF THE TCR-DELTA SEQUENCES IN PATIENT NR 5

Fig 5. Comparison of MRD detected by IgH and TCRβ-derived probes, showing evidence of clonal evolution in the IgH genes. Lanes and dots 1 to 6 correspond to PCR performed on dilutions of the initial sample (1:1, 1:10, 1:10², 1:10³, 1:10⁴, 1:10⁵). BC (for Buffy Coat) and H₂O refer to the negative controls of the PCR procedure. Lanes and dots 7 to 11 represent the PCR follow-up of the patient at different times of the clinical course. (A) displays the results obtained using IgH primers and the IgH clonogenic probe; (B) the follow-up using a TCRβ-derived clonogenic probe, performed on the same BM samples. Lanes and dots 9, 10, and 11 show the more recent evolution, with the appearance of different bands on the ethidium bromide stained gel using IgH primers. These bands are not recognized by the initial IgH oligonucleotide probe (dot 11, A) whereas the TCRβ probe continues to correctly identify the residual leukemic cells (dot 11, B).

Whether a possible focal nature of residual disease, in some cases, will hamper the use of any of these follow-up methodologies, is impossible to tell from our data or from the literature. Only very large studies performed over long periods of time will answer these questions.

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Long-term follow-up of residual disease in acute lymphoblastic leukemia patients in complete remission using clonogeneic IgH probes and the polymerase chain reaction

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