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MEASUREMENT OF RESIDUAL LEUKEMIA DURING REMISSION IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Background Complete remission of B-precursor acute lymphoblastic leukemia (ALL) has traditionally been defined as the near absence of lymphoblasts in a light-microscopical examination of stained bone marrow smears, but a patient in remission may still harbor up to 10^{10} leukemia cells. We investigated whether there is a relation between the outcome of treatment and submicroscopic evidence of residual disease.

Methods We conducted a prospective study of patients during a first clinical remission using a quantitative polymerase-chain-reaction (PCR) assay capable of detecting 1 viable leukemia cell among 200,000 normal marrow mononuclear cells and a clonogenic blast-colony assay. Bone marrow specimens from 24 children were sequentially evaluated during a five-year period, and the results were compared with the clinical outcome.

Results Seven patients relapsed and 17 remained in remission 2 to 35 months after the completion of treatment. The levels of residual leukemia-cell DNA in the two groups were significantly different ($P < 0.001$; 95 percent confidence interval for the difference in the mean log-transformed ratio of leukemia-cell DNA to normal bone marrow-cell DNA, 0.38 to 1.28). Autoregression analyses identified trends for individual patients that were associated with relapse. Despite continued remission in 17 patients, evidence of residual leukemia was detected by PCR in 15 and by both PCR and blast-colony assays in 7.

Conclusions Molecular signs of residual leukemia can persist up to 35 months after the cessation of chemotherapy in children with ALL in remission. This suggests that eradication of all leukemia cells may not be a prerequisite for cure. (N Engl J Med 1997;336:317-23.)

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HEMATOLOGIC remission in acute lymphoblastic leukemia (ALL) is defined as fewer than 5 percent lymphoblasts in a light-microscopical examination of the bone marrow. However, in patients cured of childhood ALL, leukemia cells could persist even when no lymphoblasts are visible in the marrow.¹ Results with sensitive methods of detecting leukemia cells support this idea. Estrov et al., using a clonogenic assay, unambiguously detected submicroscopic leukemia in bone marrow specimens from patients in remission.² Subsequently, with polymerase-chain-reaction (PCR) amplification of rearranged sequences of the variable region of the immunoglobulin heavy-chain (*IgH*) gene, Yamada et al. detected occult leukemia cells after 18 months of therapy in a few patients considered to be in remission.³

The detection of residual ALL by PCR led Nizet et al.⁴ to pose four questions that the technique had the unique potential to answer: What is the pattern of disappearance of leukemia cells in patients with prolonged remissions? Are sequential determinations of the levels of residual leukemia-cell DNA required to determine clinical outcome, or is there a threshold level that predicts relapse? Can patients be considered cured despite the presence of persistent leukemia cells?

To answer these questions, and in so doing establish the relation between treatment outcome and sub-

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microscopic evidence of residual disease, we initiated a prospective study of 24 children with B-precursor ALL using quantitative PCR⁵ and clonogenic blast-colony assays.⁶ We found evidence of residual leukemia in 15 of 17 patients who remained in prolonged remission after the completion of treatment.

METHODS

Study Subjects

Between September 1991 and November 1993, 74 previously untreated patients with B-precursor ALL began treatment according to an institutional protocol (P89-04).⁷ Remission-induction therapy consisted of four weeks of treatment with vincristine, prednisone, and asparaginase. Regimens for continuation therapy consisted of combinations of methotrexate, mercaptopurine, cytarabine, etoposide, and cyclophosphamide. Intrathecal methotrexate, hydrocortisone, and cytarabine were administered weekly during induction therapy and every six weeks thereafter. Treatment was continued for 18 to 24 months after complete remission was induced. The last 25 consecutively accrued subjects in the trial were prospectively evaluated for residual leukemia, after informed consent was obtained; these 25 make up the study population for the investigation described here. The data analysis reported here was completed on April 1, 1996. The study was approved by the institutional review board of the M.D. Anderson Cancer Center.

Bone Marrow Specimens

The diagnosis was based strictly on the light-microscopical appearance of bone marrow smears, and remission was defined as a finding of less than 5 percent blasts in a cellular marrow specimen.⁸ Cytogenetic studies and immunophenotyping were routinely performed during the diagnostic evaluation. All diagnostic bone marrow specimens contained more than 90 percent HLA-DR+, CD19+ cells.

During clinical remission, bone marrow aspirates were obtained at the end of induction therapy and, when possible, every three months thereafter. An average of 10 ml was aspirated into a heparin-treated syringe. Mononuclear cells were isolated, and contaminating T cells and B cells with surface immunoglobulins were removed.^{5,6}

PCR Amplification of Rearranged *IgH* Genes

Special precautions were taken to prevent contamination during the PCR procedures.⁹ The rearranged *IgH* gene from leukemia cells obtained at diagnosis and bone marrow samples obtained during remission was amplified by a previously described method.^{5,6,10} To be considered positive, a reaction had to have a band of appropriate mobility detected by ultraviolet illumination of an ethidium bromide-stained gel. As confirmation, positive bands were excised from the gel, purified, and sequenced. The use of patient-specific primers and sequencing minimized the possibility of technical artifacts.

Quantitation of Residual Disease by PCR

The amount of residual leukemia was determined with a limiting-dilution method.⁵ Strict adherence to this method has yielded estimates of residual disease with a standard deviation of 0.25 log₁₀ (the ratio of leukemia-cell DNA to normal bone marrow-cell DNA); separate dilution studies with leukemia-cell DNA from patients with newly diagnosed disease⁵ consistently demonstrated a threshold for detection of about 5×10⁻⁶. Because there was no reliable method of quantitation during the first year of this study, stored DNA from samples taken during this period was later reanalyzed by quantitative PCR whenever possible. However, PCR results for 25 samples (12.5 percent of all samples) obtained from eight patients during the first 12 months of

treatment could not be reanalyzed, so only qualitative results were recorded in these instances.

Blast-Colony Assay

The primary and self-renewal colony-culture assays, in which the leukemic nature of the cells was verified by PCR and sequencing techniques, have been reported previously.^{2,6} The blast-colony assay verifies the presence of viable leukemia cells in the sample but does not quantitate residual leukemia. Cells are first cultured for five to seven days before their self-renewal capability is assessed by disrupting the colonies in the initial culture and replating them.⁶ After a further five to seven days of incubation, individual colonies are microaspirated, and PCR amplification of the DNA from the colonies followed by sequencing is then performed. We demonstrated a high degree of correlation (P<0.001; interclass correlation coefficient, 0.46) between the results of this assay and the presence of residual disease detected by PCR in marrow samples obtained during treatment for the patients in this study.¹¹ The false negative rate for the blast-colony assay was 22 percent in the case of residual-disease levels estimated to be less than 0.001 by PCR.

Statistical Analysis

Fisher's exact test was used to measure association and independence in contingency tables. The analysis of the length of time to relapse used standard Kaplan-Meier methods. Two-way analysis of variance for the relapse and remission groups, with time analyzed in six-month intervals, was performed on both the DNA ratio (ratio of leukemia-cell DNA to normal bone marrow-cell DNA) ranked in order from lowest to highest and the log-transformed DNA ratio. Residual-disease trends were analyzed with autoregression models that estimated the upper bounds of the 95 percent tolerance intervals from a minimum of the first three or four measurements, against which subsequent levels measured during remission were compared.¹² Levels measured during remission that exceeded these estimates were considered to indicate positive trends; patients were excluded from the analysis if these levels were recorded at the time of clinical relapse. Therefore, prediction of relapse necessitated at least one measurement of these levels during remission in addition to the initial three or four measurements required to calculate either a moving average or moving-line comparison. Since the level of detection was finite, we were unable rigorously to define a nadir or mean value at all time points. However, for trend analyses, levels below the threshold of detection were assigned a ratio of leukemia-cell DNA to normal bone marrow-cell DNA of 2.5×10⁻⁶.

RESULTS

Study Population

On the basis of their age and white-cell count at diagnosis, 15 patients treated according to protocol P89-04 were at standard risk for relapse, 7 were at intermediate risk, and 3 were at high risk, according to the prognostic factors identified by Smith et al.¹³ None of the patients were infants, and none had (4;11), (9;22), or (1;19) chromosomal translocations. Patient 7 was lost to follow-up after nine months of treatment and therefore could not be evaluated. Figure 1 shows disease-free survival for the remaining 24 patients. The ages and white-cell counts of the other 49 patients with B-precursor ALL treated according to the protocol were similar to those of the study group, and the rate of disease-free survival for all 74 subjects was 67 percent (95

percent confidence interval, 55 to 79 percent) at 36 months and 59 percent (95 percent confidence interval, 44 to 74 percent) at 48 months.

Detection of Residual Leukemia by PCR

PCR amplification of DNA from diagnostic bone marrow samples yielded at least one band in each case, corresponding to the product of 300 to 400 bp expected from rearrangements of the *IgH* gene. Thirteen patients had a single rearrangement of *IgH*, nine had two rearrangements, and two had three rearrangements. We designed patient-specific primers and amplified all the leukemia-specific rearrangements in the DNA from bone marrow specimens obtained from each patient during remission. During sequential monitoring, changes in the sequence of the leukemia-specific rearrangements were detected in three patients by sequencing the DNA isolated from leukemia-cell colonies grown in the blast-colony assay. PCR failed to amplify rearranged *IgH* sequences for only one eligible patient whose leukemia cells had an *IgH* rearrangement that was detectable by Southern blot analysis.

As additional controls for the PCR, the patient-specific primers for the index patients were used in PCR analyses of DNA from bone marrow samples obtained at diagnosis from other patients with similar rearrangements of *IgH*; conversely, the patient-specific primers from these other patients were mixed with DNA from the bone marrow sample obtained at diagnosis from each of the index patients. No amplification was observed in any of these experiments, not even in cases in which there was a high degree of homology (e.g., >80 percent) between the rearranged *IgH* sequences.

Residual Disease and Clinical Outcome

Seven patients relapsed: five had relapses in bone marrow, one had a relapse in the central nervous system with a simultaneous finding of 8 to 17 percent lymphoblasts in the marrow (morphologic estimates), and one had a relapse in both these sites with more than 90 percent blasts in the marrow. Two of the five (Patients 4 and 10) with isolated bone marrow involvement relapsed while receiving treatment, whereas the other three (Patients 2, 6, and 19) relapsed after the cessation of therapy. The two cases of relapse in the central nervous system with bone marrow involvement (in Patients 1 and 23) occurred after the completion of treatment. The risk of relapse for these seven patients was initially classified as standard in four, intermediate in two, and high in one.

For the 17 patients who remained in complete remission, the median follow-up period was 45 months (range, 31 to 56). Treatment was terminated at 20 months in one patient, whereas the others received 25 months of chemotherapy. The median

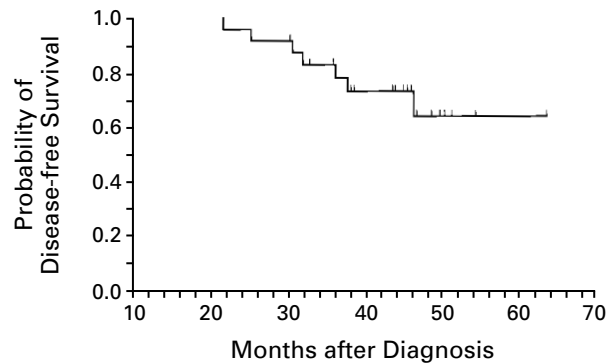


Figure 1. Kaplan-Meier Plot of Disease-free Survival, Measured from the Day of Diagnosis, in 24 Children with B-Precursor ALL Treated According to Protocol P89-04 Who Were Evaluated for Submicroscopic Residual Disease.

Tick marks designate patients who remained in complete remission at the time of the last follow-up visit.

follow-up after the completion of treatment was 19 months (range, 2 to 35); 14 patients remained without therapy during follow-up for more than 1 year.

When the two groups (the patients with relapse and those in continued remission) were compared by two-way analysis of variance that adjusted for the effect of time, the levels of residual leukemia-cell DNA, as determined by quantitative PCR, were significantly higher among patients who relapsed ($P < 0.001$) (Fig. 2). For the 17 patients who remained in remission, the fraction of positive samples and the median level of residual leukemia-cell DNA were lowest at 15 and 21 months; however, half the samples were positive during these periods (Fig. 2). The estimated mean level of residual leukemia-cell DNA at any specific time was not significantly associated with the probability of relapse (data not shown).

The levels of residual leukemia-cell DNA over time for the seven patients who relapsed are shown in Figure 3. Since results from single time points were not predictive of relapse, we sought trends (i.e., successive changes in the levels of leukemia-cell DNA) that were significantly associated with relapse. Twelve- and 20-fold increases in the levels between successive determinations were not specific for relapse, although all seven patients had 12-fold increases. These increases preceded the clinical diagnosis of relapse by four to nine months. We used autoregression analyses to improve specificity, and the best results were obtained with moving-line models.¹² A moving-line analysis fits previous measurements to a straight line that is extended to project the next data point. A value that exceeds the 95 percent tolerance interval is considered significant. With the moving-line analyses, the sensitivity increased to

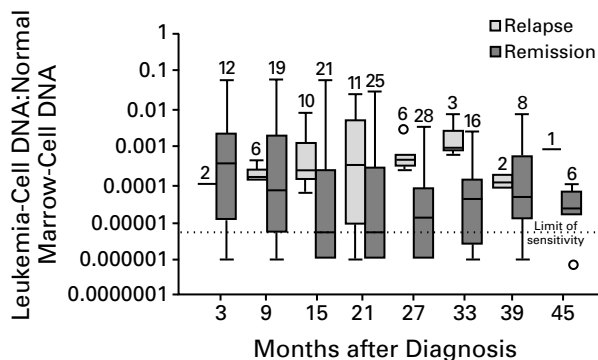


Figure 2. Median and Quartile Distributions of Residual-Disease Levels among the 7 Patients Who Relapsed and the 17 Who Remained in Remission.

The levels were measured by PCR. Negative results were arbitrarily plotted at the 10^{-6} level. The body of the box shows the central 50 percent of values, with the other quartiles at either end. For values that varied by more than 1.5 and less than 3 times the length of the box, the extreme boundaries are indicated by circles instead of bars. The number of samples analyzed in each group is shown above each box. More than one sample was obtained from many patients during the six-month period covered by each box. Two-way analysis of variance of the log-transformed DNA ratio and the DNA ratio of samples obtained during remission and ranked in order from lowest to highest showed a significant difference ($P < 0.001$) between patients who subsequently relapsed and those who remained in remission (95 percent confidence interval for the difference in the means, 0.38 to 1.28).

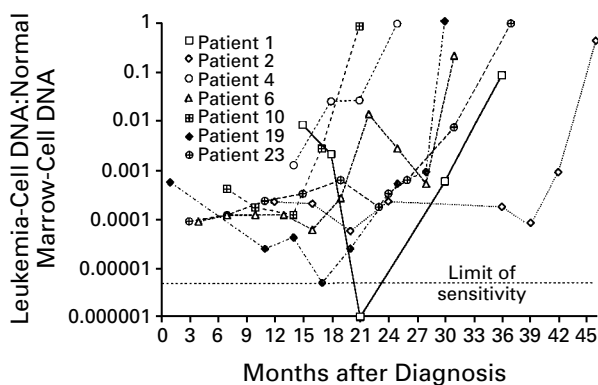


Figure 3. Residual-Disease Levels Estimated by Quantitative PCR in the Seven Patients Who Relapsed.

In each case the value of the final point correlated with the percentage of lymphoblasts found on microscopical analysis (within the standard deviation of the assay); the penultimate point was obtained during clinical remission. The limit of sensitivity is 5 leukemia cells per 1 million normal marrow mononuclear cells; the single negative PCR result was arbitrarily plotted below this level. The standard deviations for each quantitative estimate (0.25 log) are not shown, nor are the qualitatively positive PCR reactions (four samples each for Patients 1 and 2, two samples for Patient 4, and one sample for Patient 6) obtained during the first 12 months after diagnosis, before we developed the quantitative assay.

1.00 when at least four measurements were required to test a fifth or subsequent determination; however, the number of patients who relapsed and could be analyzed by this method decreased to four. The model that included at least four data points was the most sensitive and specific and predicted clinical outcome at a statistically significant level. Figure 4 shows two examples of moving-line autoregression analysis.

Presence of Residual Disease after the Completion of Treatment

Fifteen of the 17 patients who remained in remission and all 5 who relapsed after the completion of treatment had residual leukemia-cell DNA detected by PCR. In all 15 who remained in remission, at least the last sample was positive. In all but 3 of the 20 with positive PCR results, the results were positive in two or more marrow samples. The two patients in whom the PCR assay was negative had two and five negative samples.

Sufficient marrow cells obtained during remission were available to perform the blast-colony assay in 12 of the 15 patients with positive PCR results who remained in remission and 4 of the 5 patients who relapsed after the completion of therapy. A positive result was defined as one in which PCR amplification and sequencing of the DNA from the colonies revealed the *IgH* rearrangement of the leukemia clone. The assay was positive for all 4 of those who relapsed, and it was positive in at least one sample from 7 of the 12 patients who remained in remission. In the five patients with discordant results, the residual disease levels estimated by PCR were always less than 0.001. The blast-colony assay was negative in both patients with negative PCR results. Table 1 presents the results of the blast-colony and PCR assays of the 41 paired specimens obtained after the completion of therapy.

DISCUSSION

In this prospective study we used specific and quantitative PCR and clonogenic blast-colony assays to detect residual leukemia-cell DNA in 24 children with ALL who had undergone intensive chemotherapy. In patients who remained in remission, the PCR-assay values generally declined to the lowest levels during the second year of treatment (Fig. 2). However, only half the samples during this period tested negative by the PCR assay. We found evidence of residual leukemia in a substantially higher percentage of patients at all times after diagnosis than reported in previous studies.^{14,15} Two important technical factors can explain this difference. First, we used fresh, viable, purified bone marrow cells for every assay. Second, the sensitivity of our PCR was consistently greater than the ratio of leukemia-cell DNA to normal bone marrow-cell DNA of 0.001 to

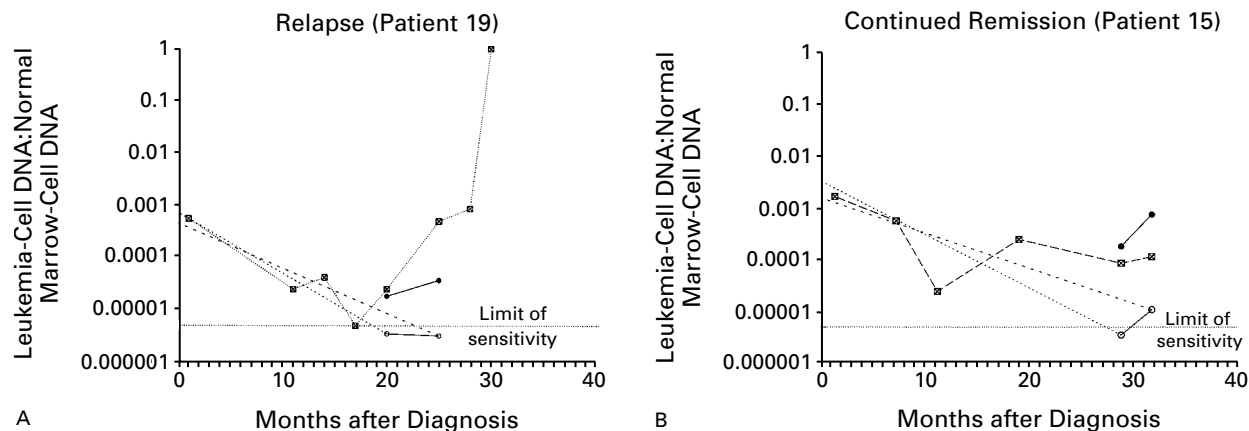


Figure 4. Examples of Autoregression Analysis with a Moving-Line Model and Four or More Data Points.

In both panels, experimental data are represented by the squares, and the broken lines represent a least-squares fit to previous data for that patient. Open circles are the projected values for the next measurement, and the solid circles the upper bounds of the 95 percent tolerance intervals corresponding to the projected values. In Panel A, the measurements at 20 and 25 months exceeded the 95 percent tolerance interval. In Panel B, the measurements at 29 and 32 months did not exceed the 95 percent tolerance interval.

0.00001 reported in the other studies; this was achieved with replicate PCR amplifications that used a minimum of 10 μ g of DNA, roughly equivalent to 2 million cells. We took extensive precautions to avoid false positive PCR results. All PCR products were sequenced to verify that the leukemia-associated *IgH* rearrangement was amplified; we detected no cross-contamination of specimens or reagents; and the results of the PCR correlated significantly with those of a clonogenic blast-colony assay.¹¹ The PCR results showed a characteristic pattern of increasing levels of residual disease in patients who relapsed, which suggests that viable cells, and not dead or dying leukemia cells, were detected by the assay.

In the 24 patients we studied, sequential determinations of residual leukemia were used to predict the clinical outcome. We could not identify a threshold value in the PCR assay that was uniformly associated with relapse. Indeed, two patients in extended remission consistently had ratios of leukemia-cell DNA to normal bone marrow-cell DNA that exceeded 0.001. A threshold may not have been detected in the first months after diagnosis because of the small number of measurements made during this period. Autoregression analyses showed a trend for increasing levels of residual leukemia-cell DNA to be significantly associated with relapse. This positive trend was present before relapse whether the patients were receiving treatment or had completed therapy, and whether they had bone marrow or central nervous system recurrences. When four determinations were required to evaluate subsequent residual-disease trends, three of seven patients who relapsed could not be evaluated, in part because of a paucity of measurements made in these patients during the

first year of the study. The sensitivity of 1.00 in the case of the other 4 relapses and the specificity of 0.88 in the case of the 17 patients who remained in remission suggest that it is possible to predict the likelihood of relapse in individual patients. Testing this hypothesis will require a large prospective study with samples taken at regular intervals. We calculate that analyses of samples obtained at the end of induction therapy and every three months thereafter could predict 90 percent of relapses that occur more than one year after diagnosis in patients treated according to current protocols.

Previous investigations have generally failed to detect residual disease by PCR after the scheduled discontinuation of therapy, except in patients who relapsed quickly.¹⁶⁻¹⁸ These results might mean that cure and eradication of leukemia cells are synonymous, since the probability of cure for patients in remission at the end of treatment exceeds 75 percent. However, our results indicate that cure and the absence of leukemia cells may not be synonymous. We detected evidence of persistent residual disease by PCR in 15 of 17 patients who remained in remission between 2 and 35 months after the cessation of therapy. The expected number of future relapses for the 15 patients with positive PCR results, estimated from the disease-free survival rate for all 74 patients treated according to the P89-04 protocol, is 1 (95 percent confidence interval, 0 to 5). In 7 of these 15 patients, each of whom had completed treatment 1.3 to 2.9 years earlier (median, 1.6), the presence of occult leukemia was verified independently by the blast-colony assay. The established relapse rate for these seven patients is 10 to 20 percent^{1,19}; the probability that all seven will relapse is 1 in 100,000 to

TABLE 1. RESULTS OF BLAST-COLONY ASSAY AND PCR FROM THE SAME SAMPLE AFTER THE COMPLETION OF THERAPY.

PATIENT NO.	MONTHS AFTER COMPLETION OF THERAPY	BLAST-COLONY ASSAY*	PCRT
Patients who relapsed			
1	0	+	2.0×10^{-3}
	3	-	$< 5.0 \times 10^{-6}$
	12	-	5.6×10^{-4}
2	18‡	-	3.6×10^{-2}
	0	+	5.6×10^{-5}
	4	-	2.2×10^{-4}
	19	+	7.0×10^{-5}
6	22	+	8.4×10^{-4}
	0	+	2.6×10^{-4}
	3	+	1.3×10^{-2}
19	9	-	5.0×10^{-4}
	12‡	+	3.0×10^{-1}
	0	-	5.0×10^{-4}
	3	+	5.6×10^{-5}
Patients who remained in remission with at least one positive blast-colony assay			
3	0	+	$< 5.0 \times 10^{-6}$
	3	-	1.3×10^{-5}
	6	+	5.6×10^{-5}
8	0	+	$< 5.0 \times 10^{-6}$
	5	-	$< 5.0 \times 10^{-6}$
	9	-	$< 5.0 \times 10^{-6}$
11	14	-	5.0×10^{-6}
	0	+	3.0×10^{-3}
	12	0	-
16	3	+	2.6×10^{-3}
	12	-	7.0×10^{-3}
	3	+	$< 5.0 \times 10^{-6}$
17	7	-	1.2×10^{-4}
	13	-	4.2×10^{-5}
18	7	+	5.0×10^{-5}
	0	+	3.2×10^{-4}
	4	-	2.0×10^{-5}
Patients who remained in remission with negative blast-colony assays			
5	0	-	$< 5.0 \times 10^{-6}$
	3	-	$< 5.0 \times 10^{-6}$
	15	-	$< 5.0 \times 10^{-6}$
9	0	-	$< 5.0 \times 10^{-6}$
	14	0	-
15	3	-	8.9×10^{-4}
	0	-	1.2×10^{-4}
20	0	-	$< 5.0 \times 10^{-6}$
	21	0	-
22	0	-	$< 5.0 \times 10^{-6}$
	0	-	$< 5.0 \times 10^{-6}$

*The blast-colony assay verifies the presence of viable leukemia cells in the sample but does not quantitate residual leukemia. Cells are first cultured for five to seven days before their self-renewal capability is assessed by disrupting the colonies in the initial culture and replating them.⁶ After a further five to seven days of incubation, individual colonies are microaspirated and PCR amplification of the DNA from the colonies followed by sequencing is then performed. Plus signs indicate positive results, and minus signs negative results.

†The values shown for PCR are the ratios of leukemia-cell DNA to normal bone marrow-cell DNA. A PCR level of less than 5×10^{-6} is below the level of detection.

‡The sample was obtained at the time of clinical relapse.

1 in 10 million. Our previous comparison of PCR and blast-colony assays showed that the probability of seven or more simultaneous false positive results from both assays is less than 1 in 10 million.

Taken together, our results challenge the dogma about the nature of cure, which is based on animal models of leukemia.²⁰ Our data imply that more than 10,000 leukemia cells may persist in a patient who remains in long-term remission (the approximate equivalent for the threshold of PCR positivity)²¹ and that the cure of ALL may not require the elimination of all leukemia cells. Nizet et al.²² and Wu et al.²³ found that only two of five and three of nine patients, respectively, with residual disease at the end of two years of treatment relapsed, but their follow-up was limited and the sample size was small.

For leukemias other than ALL, persistently positive PCR assays have been associated with relapse. However, residual disease has been observed in patients with t(8;21) acute myelogenous leukemia in long-term remission.^{24,25} Jurlander et al. have recently reported the persistence of the *AML1-ETO* fusion transcript after allogeneic bone marrow transplantation and speculate that the quantitative analysis of this t(8;21) transcript on sequential bone marrow specimens will be necessary to determine its predictive value.²⁶ We speculate that chemotherapy may “cure” patients with some forms of acute leukemia by effects other than those directly related to chemotherapy. Our data support those of Gale and Butturini,²⁷ who suggest that since maintenance chemotherapy does not eliminate all remaining leukemia cells, other processes could control the accumulation of these cells. These may include modifications to the program controlling the growth of leukemia cells, altered immune surveillance, or the interaction of the malignant cells with the microenvironment of normal bone marrow.²⁸ The identification of HLA-unrestricted cytotoxic T-cell clones specific for lymphoblasts suggests that the immune system can destroy ALL cells.²⁹ Further investigations of residual ALL at the end of treatment may allow a resolution of these issues.

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