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Early Resistance to Therapy during Induction in Childhood Acute Lymphoblastic Leukemia[1]

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ABSTRACT

Many patients with acute lymphoblastic leukemia (ALL) are not cured by current therapy because of the development of drug resistance. It is not clear when resistance develops during the growth of the leukemic clone and whether resistant cells are already present at diagnosis or develop later during treatment. Twenty-two uniformly treated children with ALL were studied throughout induction treatment. The size of the leukemic clone in blood and marrow was estimated by limiting dilution PCR analysis, using the rearranged immunoglobulin heavy chain gene as a molecular marker. The decline in the number of leukemic cells was biphasic in virtually all patients. For both marrow and blood, the logarithmic mean of the number of leukemic cells fell by approximately four orders of magnitude during the first 2 weeks, one order of magnitude during the third week, and not at all during the last two weeks of induction treatment. For marrow, the median of the fraction of leukemic cells in each patient that survived per week of treatment was 0.008 for the first 2 weeks, 0.12 for the third week, and 1.4 for the last 2 weeks; for blood, the corresponding figures were 0.003, 0.14, and 0.69, respectively. In individual patients, the results for marrow and blood showed good correlation. The biphasic decline of leukemic cell number suggests that most leukemic cells were sensitive to treatment and were rapidly killed, leaving behind a minor but substantial population of drug-resistant cells. The most likely explanation for this phenomenon is that these resistant cells were already present at diagnosis, their resistance having originated from genetic or epigenetic mutations during prior growth of the leukemic clone.

INTRODUCTION

Childhood ALL[3] has acted as a successful model for the drug treatment of cancer in general, as a number of the principles developed for successfully treating this disease have been subsequently applied to many different forms of cancer. Drug resistance remains a major ongoing problem in ALL, because many patients, particularly adults, are still not cured by chemotherapy. Drug resistance is *ipso facto* present at the time of relapse, but its presence at this time does not indicate when or how it has developed. Two hypotheses have been suggested to account for drug resistance: (a) that the genetic changes, most likely mutations, responsible for resistance occur early, during growth of the leukemic clone, so that some resistant cells are already present at diagnosis; and (*b*) that the genetic changes occur later, during treatment, possibly as the result of the mutagenic effect of cytotoxic drugs and radiation. These two hypotheses are not mutually exclusive but they do suggest different approaches to treatment.

The early-mutation hypothesis predicts that the leukemic population at diagnosis will be heterogeneous and contain one or more subpopulations of resistant cells, whereas the late-mutation hypothesis predicts that the leukemic population at diagnosis will be relatively homogeneous and that any resistant subpopulations will arise later. Drug resistance *in vivo* can be measured by the rate of decline of the leukemic population during drug treatment. We therefore used molecular techniques to study the decline of MRD during induction therapy to examine these two hypotheses.

MATERIALS AND METHODS

Patients. A cohort of 40 consecutively enrolled patients with B-lineage ALL who had been treated in the Australian and New Zealand Children's Cancer Study Group Trial VI at the Women's and Children's Hospital, Adelaide, was studied. Patients with the Philadelphia chromosome or who had not attained morphological remission at the end of one round of induction were excluded to keep the study population as uniform as possible. The patients have been described elsewhere (1). This study was approved by both the Women's and Children's Hospital and the Flinders Medical Center Ethics Committees.

Tissue Samples and DNA. Bone marrow aspirates and peripheral blood samples were obtained at diagnosis, and bone marrow, aspirates, trephines, and peripheral blood samples were obtained on days 14, 21, and 35 of induction treatment. Samples were not obtained from some patients on both days 14 and 21. Cells were removed from trephines by crushing the sample and continuously washing with saline containing 2.5 μM K_2 EDTA, as described previously (1). DNA was extracted by the standard methods of proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation.

Treatment. Systemic induction therapy consisted of daily oral prednisolone (40 mg/m^2) for 4 weeks with tailing off over the fifth week; vincristine (1.5 mg/m^2) and daunorubicin (25 mg/m^2) weekly $\times$ 4 on the first day of each week; and erwinia L-asparaginase (6000 units/m^2) three times weekly for 3 weeks. The final doses of vincristine, daunorubicin, and asparaginase were given on day 21.

Quantification of Leukemia. Leukemia was quantified by using the CDR3 region of the rearranged immunoglobulin heavy chain (IgH) gene as a molecular marker for cells of the leukemic clone (2) and the N-*ras* gene as a molecular marker for all cells. For each patient the rearranged IgH gene was sequenced, and a pair of "leukemia-specific" primers was synthesized. The numbers of amplifiable leukemic targets and amplifiable N-*ras* targets were quantified by PCR using limiting dilution analysis with Poisson statistics (3, 4), and the proportion of nucleated marrow cells that belong to the leukemic clone was calculated assuming each leukemic cell contains one rearranged IgH gene and all cells contain 2 N-*ras* genes. For samples in which leukemia could not be detected, an upper limit value for the level of MRD could be calculated from the number of amplifiable genomes studied.

Marrow. Leukemia could be quantified in 27 of the 40 patients (67.5%), the main reason for failure being technical difficulties in obtaining an IgH sequence for use as a clonal marker. Five of the 27 patients had marrow samples only at diagnosis and at the end of induction treatment and were excluded from this study because their results could provide no information on serial changes in MRD. Their response to induction appeared comparable with those of the other patients. Analysis of MRD was based on the remaining 22 patients who provided marrow samples on 57 occasions. Leukemia was detected and quantified on 51 of these (89%), and on the remaining six occasions an upper limit value for the level of MRD was estimated.

Some estimations of MRD in individual patients had been performed either in duplicate on the same sample or by studying a trephine specimen in addition to an aspirate. Duplicate estimations were pooled by calculating the geometric mean of both results or, if MRD had not been detected in one or both

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[3] The abbreviations used are: ALL, acute lymphoblastic leukemia; MRD, minimal residual disease.

estimations, by pooling the raw data and calculating the actual value for MRD or the upper limit value. Because most published MRD values are based on aspirates, trephine estimates of MRD were converted to an aspirate value by dividing the level of MRD by 4.1. This factor, which has been determined previously as representing the mean ratio between trephine and aspirate MRD levels (1), probably results from lesser contamination of trephines by peripheral blood. Pooling with actual aspirate MRD levels was then performed as described above for replicate estimations on the one sample.

Blood. Levels of leukemia at diagnosis were calculated from the white cell count and the differential count, as described previously (5). Fifteen of the 22 patients provided blood samples for study, but 2 of them never had leukemia detected in their blood and were therefore excluded from this part of the study. The remaining 13 patients provided a total of 31 blood samples after diagnosis. Leukemia was detected and quantified in 19 of these (61%), and an upper limit value estimated for the remainder.

Calculation of Absolute Levels of MRD. The cellularity of both marrow and blood is known to vary substantially during induction treatment. Because of this variation, the use of relative values for MRD, *i.e.,* leukemic cells/total cells, is misleading because it will lead to a systematic underestimation of the rate of decline of leukemia during periods when total cellularity is decreasing and a systematic overestimation during periods when total cellularity is increasing. The data for relative MRD levels were therefore converted to an absolute number of leukemic cells/unit volume by multiplying the relative MRD level and the absolute number of cells/unit volume. For blood, this was straightforward because the absolute number of leukocytes/liter was known. For marrow, cells/unit volume were estimated by counting the number of cells recovered per trephine and assuming that the mean trephine volumes at each stage of treatment remained the same. This assumption would not have biased any estimates of means, although it would have increased the variance. The logarithmic means of cellularities were 2.9×10^5 cells at day 14 ($n = 18$), 1.0×10^5 at day 21 ($n = 16$), and 6.9×10^5 at day 35 ($n = 21$; Ref. 1). An additional 13 marrows, reported as being of normal cellularity, were obtained from patients 1–2 years after diagnosis, and the logarithmic mean of cells recovered from the trephines was 3.2×10^6. No marrow trephines were available at diagnosis. Because the marrow at diagnosis is usually judged to be "packed" with leukemic cells, we assumed that the cellularity at diagnosis was $2 \times$ normal. We also assumed that the mean cellularity of the year 1–2 marrows was $1 \times$ normal. Both of these assumptions, almost certainly underestimated the true differences from normal values; if so, they were conservative, tending to act against the detection of significant differences.

Calculation of the Rate of Decline of the Leukemic Clone during Early and Late Induction. The rate at which the leukemic clone declined was expressed as the fraction surviving per week (*FS/W*) during treatment. This value is given by: $FS/W = \sqrt[w]{N_2/N_1}$, where N_1 and N_2 are the numbers of leukemic cells at the beginning and end of a time of w weeks. N_1 and N_2 can be expressed as absolute numbers or as fractions of day 0 values. It should be noted that the actual volume of marrow or blood used for estimation of cells/unit volume does not affect the final calculation, because it is present in both numerator and denominator and cancels out.

Actual or limit values for *FS/W* were calculated between day 0 and day 14, between day 14 and day 21, and between day 21 and day 35 by assuming a constant exponential rate of decline between the two time points of interest and calculating the *FS/W* either from two actual values or from one actual value and one upper limit value.

Statistics. The significance of observed differences in *FS/W* for different time intervals were tested using the two-tailed Mann-Whitney *U* test, unpaired except where indicated. For some samples, particularly blood, only an upper limit value could be estimated for the level of MRD, and thus only an upper or lower limit value could be calculated for *FS/W*. For such samples the true value of MRD lies somewhere between the upper limit value and zero. Discarding such samples or equating the true value to the upper limit value would introduce a systematic upward bias, whereas equating the true value to zero would introduce a systematic downward bias. For this reason, we made assumptions as to the true MRD values for samples with upper limit values and performed a sensitivity analysis to test the effects of varying these assumptions. If an upper limit value was followed by a later actual MRD value that was greater than that upper limit value, suggesting that the leukemic population had not declined between the two time points, we regarded the level of MRD at the earlier time point as being $0.5 \times$ the upper limit value; otherwise we regarded the level of MRD as being $0.1 \times$ the upper limit value.

Sensitivity Analysis. The assumptions made with regard to marrow cellularity and upper limit values would affect the significance values obtained from testing observed differences. A sensitivity analysis was therefore performed by varying the assumptions to observe the effect on significance values and on conclusions drawn from them. The assumed relationship to normal cellularity for day 0 and year 1–2 marrows affects only the *FS/W* calculated for days 0–14, because the *FS/W* calculated for the other time intervals is based on actual measurements. Significance values were therefore also calculated, assuming that the cellularity on day 0 was equal to the cellularity of normal marrow. For the transformations of upper limit values, the assumed values of MRD were varied upwards or downwards within wide limits, *FS/W* was calculated, and significance testing was performed.

RESULTS

Table 1 shows, for each patient, the level of leukemia in marrow and blood at various times during induction. The relative MRD levels

Table 1 *Level of leukemia in individual patients at various stages during induction, measured as the fraction of leukemic cells surviving in marrow (relative to diagnosis) and the absolute number of leukemic cells in blood*

	Marrow: Fraction surviving			Blood: Blasts/μl			
Patient	D14	D21	D35	D0	D14	D21	D35
464	1.2×10^{-4}	9.5×10^{-6}	9.3×10^{-7}	$<3.0 \times 10^{1}$	4.6×10^{-1}	2.9×10^{-1}	1.3×10^{-2}
467	3.2×10^{-5}	1.8×10^{-7}	2.5×10^{-6}	3.0×10^{1}		$<6.2 \times 10^{-3}$	$<1.9 \times 10^{-3}$
470	3.3×10^{-3}	7.8×10^{-5}	5.2×10^{-5}	6.0×10^{2}		3.1×10^{-2}	1.1×10^{-2}
473	1.1×10^{-3}	5.8×10^{-4}	3.4×10^{-5}	5.0×10^{5}	1.0×10^{1}	1.2×10^{1}	
474	9.0×10^{-6}	1.1×10^{-6}	7.2×10^{-6}	8.1×10^{4}	4.8×10^{-2}	1.8×10^{-2}	
475	2.9×10^{-3}		4.3×10^{-4}				
476	2.7×10^{-5}		3.6×10^{-5}				
477	8.0×10^{-4}		1.7×10^{-2}				
480	$<4.7 \times 10^{-4}$		1.6×10^{-3}				
481	3.7×10^{-6}	9.4×10^{-7}	3.5×10^{-6}	2.2×10^{3}		$<1.4 \times 10^{-1}$	1.2×10^{-1}
485	7.7×10^{-4}		7.2×10^{-7}				
489	2.0×10^{-6}	1.3×10^{-6}	3.4×10^{-7}	5.7×10^{3}	$<1.4 \times 10^{-2}$	$<9 \times 10^{-3}$	
490	1.2×10^{-5}		$<1.3 \times 10^{-4}$				
491	1.8×10^{-4}		7.9×10^{-5}				
498	1.9×10^{-6}	2.7×10^{-7}	1.7×10^{-5}	1.3×10^{4}	5.5×10^{-3}	$<1.7 \times 10^{-2}$	
499	4.3×10^{-6}	$<1.3 \times 10^{-7}$	2.1×10^{-6}	2.1×10^{5}	3.3×10^{-2}	1.9×10^{-2}	$<6.9 \times 10^{-3}$
500	3.8×10^{-7}	$<2.5 \times 10^{-7}$	1.1×10^{-6}				
501	1.7×10^{-2}	7.5×10^{-4}	2.8×10^{-3}	6.1×10^{3}	1.3×10^{1}	1.3×10^{0}	6.2×10^{-1}
502	2.5×10^{-4}	4.7×10^{-7}	$<1.1 \times 10^{-7}$	$<2.0 \times 10^{1}$	3.1×10^{-2}	$<3.5 \times 10^{-2}$	1.3×10^{-2}
505		9.4×10^{-8}	$<3.7 \times 10^{-7}$				
507		6.5×10^{-5}	8.1×10^{-6}	1.2×10^{3}		$<8.9 \times 10^{-3}$	$<2.2 \times 10^{-2}$
508	6.2×10^{-7}	1.0×10^{-7}	2.0×10^{-7}	2.9×10^{5}	3.1×10^{-2}	$<4.4 \times 10^{-2}$	$<1.8 \times 10^{-2}$

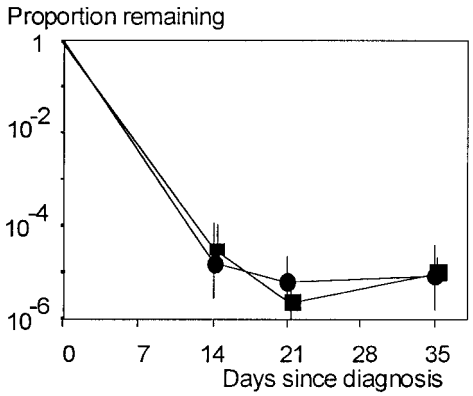

Fig. 1. Level of leukemia (actual and upper limit detection; means; *bars*, ±1 SE) in marrow (■, all samples) and blood (●, all samples).

for marrow were first converted to absolute numbers of leukemic cells per unit of volume and then normalized by dividing by the day 0 numbers; the values for blood are absolute cell numbers/liter. The means ± 1 SE on different days for the normalized levels of MRD in marrow and blood are shown in Fig. 1. For days 14, 21, and 35, the respective values were: for marrow, 5.2×10^{-5} (± 1 SE, 9.9×10^{-5} to 2.7×10^{-5}), 2.2×10^{-6} (4.9×10^{-6} to 9.5×10^{-7}), and 9.9×10^{-6} (2.1×10^{-5} to 4.8×10^{-6}); and for blood, 1.7×10^{-5} (1.1×10^{-4} to 2.5×10^{-6}), 5.7×10^{-6} (2.0×10^{-5} to 1.6×10^{-6}), and 7.4×10^{-6} (3.7×10^{-5} to 1.5×10^{-6}). The results indicate a rapid decline in leukemia during the first 2 weeks of treatment, a slowing of decline during the third week, and a plateau during the fourth and fifth weeks. The rates of decline in individual patients are shown in Fig. 2, which shows the *FS/W* of the leukemic clone in marrow and blood between days 0 and 14, days 14 and 21, and days 21 and 35. Because of the missing samples, in several patients *FS/W* could not be determined for these intervals, but data were available for days 0–21 or days 14–35, and these are also shown.

For marrow, the median *FS/W* was −2.1 log units (0.008) for days 0–14, −0.91 log units (0.12) for days 14–21, and 0.15 log units (1.4) for days 21–35. The day 0–14 results were significantly less than the day 14–21 results ($P = 0.0007$), and the day 21–35 results ($P < 0.0001$), and the day 14–21 results were significantly less than the day 21–35 results ($P = 0.0003$). For blood, the median *FS/W* was −2.6 log units (0.003) for days 0–14, −0.85 log units (0.14) for days 14–21, and −0.16 log units (0.69) for days 21–35. The day 0–14 results were significantly less than the day 14–21 results ($P = 0.008$) and the day 21–35 results ($P = 0.0008$), and the day 14–21 results were significantly less than the day 21–35 results ($P = 0.02$). There were 13 patients for whom serial marrow estimations on all 4 days were available. The within patient *FS/W* for days 0–14 was significantly different from that for days 14–21 (median difference, −1.2 log units; $P < 0.02$, paired Mann-Whitney test), which in turn was significantly different from that for days 21–35 (median difference, −1.3 log units; $P < 0.001$, paired Mann-Whitney test).

The sensitivity analysis confirmed the reality of the differences between the *FS/W* for the different time intervals, because significant differences persisted, despite wide variations in the magnitude of the assumptions used. For marrow, the differences between the results for the three time intervals remained significant when cellularity on day 0 or day 35 was equated to the cellularity of normal marrow (*P* between 0.02 and 0.001); when all assumed MRD values were equated to the observed upper limit values (*P*s between 0.01 and 0.0003); and when the assumed values for marrow MRD were decreased to any extent, even to zero (*P*s between 0.01 and 0.0003). For blood, the differences between the various time intervals remained significant until assumed MRD values were decreased by a further two orders of magnitude, at which point only the difference between day 0–14 and day 21–35 values remained significant ($P = 0.04$). Thus, the results for both marrow and blood indicate that for each of the first 2 weeks of chemotherapy, the number of leukemic cells at the end of each week was ~1% of the number at the beginning of the week, for the third week the number was ~10%, and for the fourth and fifth weeks this number was approximately unchanged.

Fig. 3 shows *FS/W* for marrow and blood when data were available for both tissues in a patient for the same time interval. Where an actual fractional survival could be calculated in both tissues, the data for marrow and blood show excellent correlation ($r = 0.91$; $n = 14$; $P < 0.001$).

DISCUSSION

The results indicate that the decline in the number of leukemic cells during induction treatment is biphasic in virtually all patients with childhood ALL. The logarithmic mean level of MRD in marrow decreased rapidly, by 4.2 logs, between day 0 and day 14, and then decreased more slowly, by 0.91 logs between days 14 and 21. In at least 19 of the 22 patients, the *FS/W* ceased to decrease between day 14 or day 21 and day 35, and the logarithmic median *FS/W* during this late phase was approximately zero. The results of measuring MRD in blood, although involving fewer observations, were very similar.

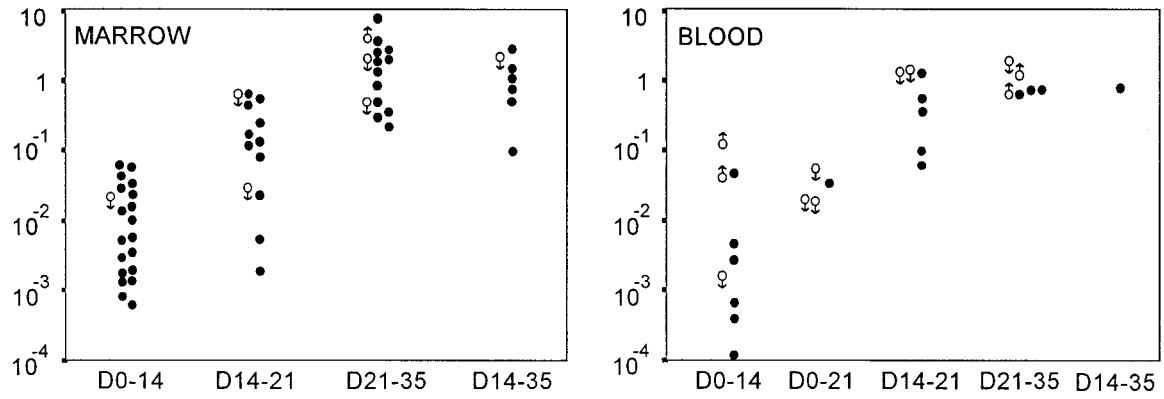

Fig. 2. The rate of decline of the leukemic population in marrow and blood for each patient during various time intervals. This is expressed as the fraction of leukemic cells that survive per week of treatment (*FS/W*) as calculated from the level of leukemia in and the time interval between two consecutive tissue samples. ●, *FS/W* calculated from two actual measurements of levels of leukemia; ♀, an upper limit was estimated for *FS/W*, because leukemia was detected only in the first tissue sample; ♂, a lower limit was estimated for *FS/W* because leukemia was detected only in the second.

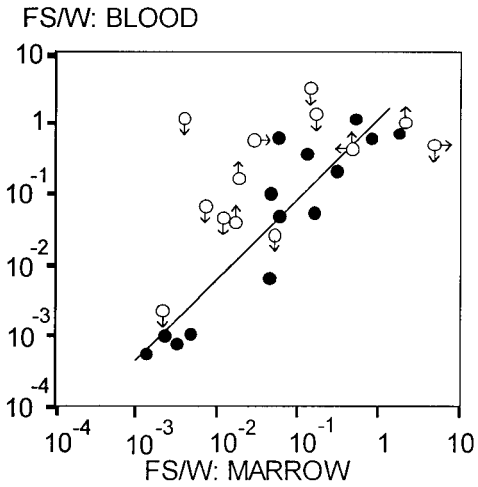

Fig. 3. Comparison of the response of leukemia to treatment in blood and marrow. Response is expressed as the fraction of leukemic cells that survive/week of treatment (*FS/W*), and each *point* shows the *FS/W* in blood and marrow for the same time interval in one patient. ●, *FS/W* calculated in absolute terms for both tissues. ○, data where an upper or lower limit was calculated for *FS/W* for one tissue (*single arrow*) or two tissues (*two arrows*). *Arrows pointing down* or to the *left* indicate an upper limit in blood or marrow, respectively; and *arrows pointing up* or to the *right* indicate a lower limit in blood or marrow, respectively. The regression line is $y = 1.14X + 0.072$.

There appear to be two possible explanations for the slowing rate of decline between days 14 and 21. Response to treatment may have been decreasing uniformly in all patients or, alternatively, some patients may have stopped responding to treatment by day 14, whereas others continued to show some response until day 21. The results in Table 1 and Fig. 2 provide some support for this latter possibility. In either event, by day 21 the population of leukemic cells appeared to be completely resistant to therapy with the four agents being used. If it is assumed that at diagnosis 10^{11} to 10^{12} leukemic cells are present in the patient with ALL, then the mean size of the resistant population can be estimated as 10^6 to 10^7 cells, a substantial number.

These observations are not consistent with the concept that the leukemic population is a homogeneous and unchanging collection of cells with a constant fraction being killed by each administration of chemotherapy, so that the rate of decrease is constant. They suggest that the biology of leukemia during treatment is more complex and that response to therapy changes with time.

Several factors may contribute to this complexity and changing response: (*a*) chemotherapy may directly mutate genes affecting drug resistance, but both the time delay between the occurrence of a mutation and its phenotypic expression, and the magnitude of the resistant population, make this explanation unlikely; (*b*) chemotherapy may lead to demethylation of the controlling regions of these genes and produce "epigenetic" mutations, but similar quantitative considerations make this possibility also unlikely; (*c*) quite possibly, chemotherapy may lead to increased expression of key genes in the leukemic cell by classical induction mechanisms. Such key genes might, for example, include genes controlling one or more of the following: proliferation, differentiation, apoptosis, drug transport, or drug metabolism; and (*d*) chemotherapy may lead to changes in expression of genes in nonleukemic cells, such as cells that are important in the detoxification or excretion of cytotoxic drugs.

Alternatively, the leukemic population evident after days 14–21 could already be present at diagnosis as one or more subpopulations that have various degrees of intrinsic drug resistance and that are selected out by therapy. Such subpopulation(s) could be resistant as the result of increased expression of nonmutated key genes but are more likely to have arisen as the result of genetic or epigenetic mutations that affect genes related to drug resistance and that result in subclonal evolution during growth of the leukemic clone. In their classic study, Luria and Delbruck (6) showed that apparent development of antibiotic resistance in bacterial populations was attributable to the selection of preexisting rare cells that had already mutated to antibiotic resistance prior to any antibiotic exposure and whose number was determined by the mutation rate and the point in time at which the mutation occurred. Goldie and Coldman (7) applied these concepts to cancer and considered the consequences of the presence of rare drug-resistant cancer cells. However, our data suggest that an additional factor may be present in leukemia and, by extension, in other cancers. Many of the mutations important in development or progression of leukemia are likely to affect genes involved in apoptosis. Such mutations will be selected for as they will lead to a survival advantage of and an increased mutation rate in the cells in which they occur, but they may incidentally result in drug resistance. As a consequence, a sizable population of drug-resistant cells is likely to develop within the leukemic population, before any exposure to drug. A previous study of three leukemic cell lines growing *in vitro* showed a mutation frequency of 3.1×10^{-5} to 1.1×10^{-3} during continuous culture and a mutation rate of $0.5–6.7 \times 10^{-6}$ per cell division (8). These values are quite consistent with the frequency of resistant cells that we observed *in vivo*.

These various inductive or selective mechanisms are not mutually exclusive and, on the evidence available, it is difficult to decide between them. There are, however, a number of circumstantial pieces of evidence suggesting that genetically resistant cells may be present at diagnosis and selected out by therapy: (*a*) the leukemic population in ALL is often genetically heterogeneous at diagnosis as evidenced by the presence of multiple immunoglobulin gene rearrangements (9). Although gene rearrangements are mutations that are selectively neutral, the coexistence of several large subclones within the leukemic population is difficult to explain unless one postulates that, except for the founder clone, there is within each subclone a dominant subsubclone that has resulted from a mutation providing a selective growth advantage; (*b*) the wide range in the size of the resistant population in different patients (Table 1) is well explained by Luria-Delbruck kinetics; (*c*) the mutation rate of leukemic cells is consistent with the observed frequency of drug-resistant cells; (*d*) at least two patients have been observed that in each of whom there were two genetically distinguishable leukemic populations present at diagnosis, a major one that was drug sensitive and a minor one that was drug resistant (10, 11); (*e*) several patients have been reported in whom there was, at diagnosis, a minor subpopulation of leukemic cells that contained a mutation of p53, which presumptively was associated with drug resistance (12, 13).

Our observations on the kinetics of leukemia during induction therapy may explain several other observations on the biology of leukemia during treatment:

(*a*) Although most children with ALL are eventually cured, residual leukemia may be detected for quite long periods during postinduction treatment (14, 15). Although other explanations for this phenomenon are possible, one explanation is that a population of leukemic cells that shows resistance to the four drugs used during induction is likely to show cross-resistance to at least some of the drugs used subsequently and to therefore decline in number only slowly.

(*b*) Many workers have now observed that the size of the leukemic population at or shortly after the end of induction is a very strong predictor of cure or relapse (16–24). The present study would interpret these observations as indicating that some cells resistant to the induction drugs are present in all or nearly all individuals and that it is the size of this resistant population, not its presence or its rate of decline, that determines outcome.

(*c*) Patients with a large percentage of blasts visible in the marrow on day 7 or day 14 are well recognized as having a poorer prognosis. This observation is often interpreted as indicating that such patients show a slower initial rate of decline of leukemia and that this slower rate of decline persists during subsequent treatment and leads to a poorer outcome. Our results suggest another interpretation, *i.e.*, that if a large resistant population is present in a patient, then it may be the dominant population as early as day 14 or even day 7 and be responsible for a high blast percentage.

How might the size of the resistant population at the end of induction determine clinical outcome? Cure by chemotherapy depends on elimination of the leukemic clone before the development of completely drug-resistant cells. Complete drug resistance is likely to be multifactorial. Both inductive and selective mechanisms may play a role and each class of mechanism can explain the fact that the majority of patients are cured, whereas a minority relapse. However, irrespective of mechanism, it can be predicted that the size of the leukemic population at the end of induction will be an important determinant of the probability that completely drug-resistant cells are already present or will develop subsequently. Mutations are likely to play a necessary, even if not sufficient, role in complete drug resistance in most patients destined to relapse. The probability that mutations occur within a population is directly related to its size, its mutation rate, and the time for which it proliferates. Thus, a large "partially resistant" population is more likely than a small one to already contain or to develop the further mutations which confer complete resistance. Furthermore, the mutations of importance are more likely to occur before diagnosis, as the partially resistant population has been growing for some time, rather than during treatment, even though the mutation rate may have been increased by chemotherapy. We therefore suggest that the potential for cure with conventional therapy is usually already determined at diagnosis and depends on whether there are leukemic cells that already contain mutations that by themselves, or together with subsequent induced changes in gene expression, lead to complete drug resistance. The early presence of completely resistant leukemic cells may well explain the observation that high levels of MRD persisting in the early months of postinduction therapy indicate a very high probability of eventual relapse (23, 24).

Our findings also have implications for treatment of ALL. They suggest that the only effect of continuation or escalation of the dose of the same four induction drugs after day 14 is to diminish even further the size of the sensitive population. This effect is of lesser importance because the sensitive population has become the minor population. Depending on the mechanism(s) responsible for our findings, there may also be implications for the nature and/or scheduling of drug therapy.

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