In Vitro Drug Sensitivity of Cells From Children With Leukemia Using the MTT Assay With Improved Culture Conditions

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The knowledge about drug resistance in childhood leukemias and acute lymphoblastic leukemia (ALL) in general is limited. This is because of the lack of a suitable in vitro drug sensitivity assay, which is in part due to low in vitro ALL cell survival. We recently adapted the highly efficient 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay to test cells from ALL patients and showed that its results were comparable with those of the DiSC assay, up to now the most valid but laborious assay. In this study, in vitro drug sensitivity was assessed in cells from 82 children with leukemia, 79 of whom had ALL, with the MTT assay. Dose response curves were obtained for 6-mercaptopurine, 6-thioguanine (6-TG), prednisolone (Pred), daunorubicin (DNR), vincristine (VCR), cytosine arabinoside (Ara-C), L-asparaginase (L-Asp), mafosfamide, and mustine. A cytotoxic effect of methotrexate could be detected in only a few cases. Large interindividual differences in drug sensitivity were detected. Compared with leukemia cells from newly diagnosed patients, leukemia cells from relapsed patients were significantly more in vitro resistant to 6-TG, Pred, Ara-C, mafosfamide and mustine but not to DNR, VCR, and L-Asp. Improvements of culture medium and methods to increase MTT reduction were studied. From 10 components tested, addition of insulin and bovine serum albumin to serum-containing medium improved ALL cell survival. Addition of succinate did not increase the amount of MTT reduction. We conclude that the in vitro MTT assay highly facilitates large-scale studies on drug resistance of ALL patients that can lead to rational improvements in existing treatment protocols.

IN VITRO DRUG sensitivity studies of acute lymphoblastic leukemia (ALL) have been performed mainly on established cell lines. However, to increase the knowledge of mechanisms and patterns of resistance involved in clinical resistance, it is necessary to study patient samples. With regard to correlation with clinical response, the most valid test of different assays, the differential staining cytotoxicity (DiSC) assay is considered to be the most suitable assay for determining drug resistance of the leukemia-lymphoma population.[1-4] However, this assay is time consuming and subject to observer error.

Mosmann[5] developed an efficient assay, based on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to a formazan product by living but not by dead cells, that can be used for large-scale studies. After the assay had been applied to chemosensitivity testing of cell lines[6-11] we adapted it for testing fresh and cryopreserved cells from ALL patients[12] and showed that MTT and DiSC assays gave comparable results.[13] Experience with in vitro chemosensitivity testing of childhood ALL is limited because of low in vitro cell survival and lack of a suitable assay. This has resulted in a dearth of drug resistance data in this type of cancer. In this article, in vitro drug sensitivity was assessed in 82 children with leukemia using the MTT assay with a panel of 10 drugs. We also studied improvements of the culture medium for ALL cells.

MATERIALS AND METHODS

Cells. Cells from 87 bone marrow (BM) and peripheral blood (PBL) samples from 82 leukemic children taken for routine diagnostic procedures were used. Seventy-five were patients with newly diagnosed ALL and seven were examined only at relapse. In 69 cases cells were tested after cryopreservation in RPMI 1640 with 10% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO). Drug sensitivity results are not influenced by cryopreservation.[13] Mononuclear cells were isolated by centrifugation (500*g* interphase, 25 minutes) with 1.077 g/mL Ficoll Isopaque (Lymphoprep, Nyegaard, Oslo, Norway). After isolation, the cells were washed two times in Earle's balanced salt solution (NPBI, Emmer-Compascuum, The Netherlands) containing 0.1% bovine serum albumin (BSA; Organon Teknika, Oss, The Netherlands) with 10-minute periods of centrifugation at 300*g*. Immunological phenotyping was done as described before.[14] Table 1 shows characteristics of the patient samples. One patient whose leukemic cells expressed surface immunoglobulins was classified as having B-ALL. Sixty-eight less mature ALL cases of B-cell lineage were defined as precursor B-ALL. This precursor B group consisted of 6 pro-B (CD10 negative, cytoplasmic μ negative), 39 early pre-B (CD10 positive, cytoplasmic μ negative), and 23 pre-B ALL (cytoplasmic μ positive) cases. There were 9 cases of T-ALL, 1 acute undifferentiated leukemia, 1 acute nonlymphocytic leukemia (ANLL), 1 mixed ALL/ANLL, and 1 chronic myelogenous leukemia (CML).

In vitro drug sensitivity assay. 6-Thioguanine (6-TG) and 6-mercaptopurine (6-MP) were obtained from Sigma; Prednisolone (Pred), methotrexate (MTX), daunorubicin (DNR), L-asparaginase (L-Asp), mustine hydrochloride (Must), cytosine arabinoside (Ara-C) and vincristine (VCR) from our hospital pharmacy. 6-MP and 6-TG were dissolved in 0.04 N NaOH, Pred in saline, and DNR, L-Asp, and Must in distilled water. At this final culture concentration, NaOH has no significant effect on pH of the medium and the viability of ALL cells in culture.[12,15] Ara-C, MTX, and VCR were obtained in soluble form. Mafosfamide (Mafos), an activated derivative of cyclophosphamide, kindly provided by ASTA Pharma Aktiengesellschaft (Z7557; Dr M. Peukert, Bielefeld, Germany) was dissolved in PBS. All drugs were further diluted in RPMI 1640 (Dutch modification, Sigma). Making up fresh solutions each time is not feasible for large-scale patient studies.[15,16] Therefore, microcul-

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Table 1. Characteristics of Patient Samples

Sample No.	Sample f/c	Immunophenotype	Initial/ Relapsed	% Malignant Cells	PBL/BM	Control Culture OD Mean	Control Culture OD CV (%)
1	c	CML	Initial	85	PBL	0.618	6
2	f	AML	Initial	84	BM	0.448	5
3	c	AUL	Initial	90	PBL	0.135	6
4	c	Pro-B ALL	Initial	99	PBL	0.366	8
5	c	Pro-B ALL	Initial	99	BM		
6	c	Pro-B ALL	Initial	97	PBL	0.161	6
7a	f	Pro-B ALL	Initial	97	BM	0.281	7
7b	f	Pro-B ALL	Initial	76	PBL	0.177	15
8	c	Pro-B ALL	Initial	99	BM	0.266	4
9	c	Pro-B ALL	Initial	80	PBL	0.380	6
10	c	Early pre-B ALL	Initial	81	PBL	0.099	24
11	c	Early pre-B ALL	Initial	89	PBL		
12	c	Early pre-B ALL	Initial	90	PBL		
13	c	Early pre-B ALL	Initial	88	PBL	0.232	6
14	c	Early pre-B ALL	Initial	94	PBL		
15	c	Early pre-B ALL	Initial	79	PBL	0.122	20
16	c	Early pre-B ALL	Initial	94	BM		
17	c	Early pre-B ALL	Initial	83	PBL	0.077	9
18	c	Early pre-B ALL	Initial	98	BM	0.164	9
19	c	Early pre-B ALL	Initial	92	PBL		
20	c	Early pre-B ALL	Initial	97	PBL		
21	c	Early pre-B ALL	Initial	83	PBL		
22	c	Early pre-B ALL	Initial	94	PBL	0.069	8
23	c	Early pre-B ALL	Initial	96	BM	0.089	6
24	c	Early pre-B ALL	Initial	98	PBL		
25	c	Early pre-B ALL	Initial	95	BM	0.064	12
26	c	Early pre-B ALL	Initial	97	BM		
27	c	Early pre-B ALL	Initial	97	BM	0.156	6
28	c	Early pre-B ALL	Initial	95	BM	0.111	9
29a	c	Early pre-B ALL	Initial	95	PBL	0.079	9
29b	c	Early pre-B ALL	Initial	99	BM	0.068	21
30	c	Early pre-B ALL	Initial	98	BM		
31	c	Early pre-B ALL	Initial	82	PBL		
32	c	Early pre-B ALL	Initial	83	PBL		
33	f	Early pre-B ALL	Initial	97	BM		
34	c	Early pre-B ALL	Initial	94	PBL		
35	c	Early pre-B ALL	Initial	96	BM	0.054	7
36	c	Early pre-B ALL	Initial	98	BM	0.056	10
37	c	Early pre-B ALL	Initial	95	BM	0.108	15
38	c	Early pre-B ALL	Initial	95	PBL		
39	c	Early pre-B ALL	Initial	90	PBL	0.129	12
40	c	Early pre-B ALL	Initial	99	PBL	0.343	10
41	f	Early pre-B ALL	Initial	91	BM	0.161	10
42	c	Early pre-B ALL	Initial	95	PBL	0.224	9
43	c	Early pre-B ALL	Initial	94	BM	0.126	9
44	f	Early pre-B ALL	Initial	97	BM	0.374	6
45	c	Early pre-B ALL	Initial	66	PBL	0.095	11

(*Continued on next page*)

ture plates are filled with 20 μL of drugs about every 2 months and stored at −20°C. Testing cells from the same patient sample twice with a 4-month interval using drugs from the same cryopreserved stock solutions resulted in similar dose-response curves and LC50 values for all drugs tested.

Cells were suspended at 2×10^6 or sometimes 1×10^6 cells/mL in RPMI 1640 containing 15% FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.125 μg/mL fungizone, 200 μg/mL gentamycin, 2 mmol/L L-glutamine, all obtained from Flow Laboratories (Irvine, Scotland), and 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite from Sigma. Eighty-microliter aliquots of the cell suspension were dispensed into 96-well round-bottomed microculture plates containing the drug dilutions. In accordance with others[17] we observed evaporation of medium in the outer wells, which were therefore filled only with medium. Column 2 contained only medium for blanking the reader. Column 3 contained cells and no drug for measuring control cell viability. Columns 4 through 11 contained six concentrations of drugs set up in duplicate.

Plates were incubated in a humidified incubator in 5% carbon dioxide for 4 days at 37°C. Then 10 μL of MTT solution (5 mg/mL MTT [Sigma] in saline stored at −20°C) was added, and after shaking for 1 minute the plate was incubated for 6 hours. Formazan

Table 1. Characteristics of Patient Samples (Cont'd)

Sample			Initial/	% Malignant		Control Culture OD	
No.	f/c	Immunophenotype	Relapsed	Cells	PBL/BM	Mean	CV (%)
46	c	Pre-B ALL	Initial	88	PBL	0.066	9
47	c	Pre-B ALL	Initial	98	PBL		
48	c	Pre-B ALL	Initial	92		0.074	22
49	c	Pre-B ALL	Initial	96			
50	c	Pre-B ALL	Initial	80	PBL	0.155	7
51	c	Pre-B ALL	Initial	78			
52	c	Pre-B ALL	Initial	96	PBL	0.232	7
53	c	Pre-B ALL	Initial	94	PBL		
54	c	Pre-B ALL	Initial	98	BM	0.090	7
55a	f	Pre-B ALL	Initial	85	PBL		
55b	f	Pre-B ALL	Initial	95	BM		
56	c	Pre-B ALL	Initial	99	PBL		
57	f	Pre-B ALL	Initial	96	BM	0.282	2
58	c	Pre-B ALL	Initial	98	BM	0.074	9
59	c	Pre-B ALL	Initial	84	PBL		
60	c	Pre-B ALL	Initial	96	BM		
61	c	Pre-B ALL	Initial	99	PBL		
62	c	Pre-B ALL	Initial	98	BM		
63	c	Pre-B ALL	Initial	100	BM	0.084	10
64	c	Pre-B ALL	Initial	96	BM	0.160	13
65	c	Pre-B ALL	Initial	72	PBL	0.132	10
66	f	Pre-B ALL	Initial	95	BM	0.397	9
67a	f	Pre-B ALL	Initial	99	BM		
67b	f	Pre-B ALL	Initial	97	PBL		
68	c	B ALL	Initial	68	PBL	0.141	8
69	c	T ALL	Initial	98	BM		
70	c	T ALL	Initial	85	PBL		
71a	f	T ALL	Initial	90	BM	0.285	4
71b	f	T ALL	Initial	91	PBL	0.188	4
72	c	T ALL	Initial	95	BM		
73	c	T ALL	Initial	85	BM	0.193	5
74	c	T ALL	Initial	67	PBL	0.127	12
75	c	T ALL	Initial	91	BM	0.085	18
76	c	Early pre-B ALL	Relapsed	97	BM		
77	c	Early pre-B ALL	Relapsed	97	BM	0.217	13
78	c	Early pre-B ALL	Relapsed	86	BM	0.090	7
79	f	Mixed ALL/ANLL	Relapsed	>65	BM	0.610	11
80	f	Pre B ALL	Relapsed	90	BM	0.701	14
81	f	T ALL	Relapsed	94	PBL	0.178	7
82	f	T ALL	Relapsed	78	PBL	0.249	6

Abbreviations: f, fresh; c, cryopreserved; PBL, peripheral blood; BM, bone marrow; control culture OD, optical density of control cells; CV, coefficient of variation.

crystals were dissolved with 100 μL of 0.04 N HCl–isopropyl alcohol (acid isopropanol), which was found to be more suitable than DMSO. The optical density (OD) of the wells was measured with a microplate reader (Titertek Multiskan MCC 340; Irvine, Scotland) at 540 nm. Round-bottomed plates can be used for OD measurements. Because 6-MP and 6-TG at high concentration have significant OD measurements, the ODs of wells containing drugs but no cells were subtracted from the ODs of corresponding wells containing cells and these drugs.[12] Leukemic cell survival (LCS) was calculated by the following equation:

$$\text{LCS} = (\text{OD treated well}/\text{mean OD control wells}) \times 100\%$$

The LC_{50}, defined as the drug concentration that results in 50% LCS, was derived by calculating the point where the dose-response curve crosses the 50% LCS point.

Medium supplements. We studied several additives to increase ALL control cell survival. Transferrin (T 5391), sodium selenite (S 9133), BSA (A 4503, fraction V), glutathion (G 6013), sodium pyruvate (S 8636), and a supplement containing insulin, transferrin, and selenium (ITS; I 1884), all obtained from Sigma, were dissolved in distilled water. Insulin (Sigma) was dissolved in Hanks' balanced salt solution, and cholesterol (C 8253, Sigma) in 96% ethanol at 50°C. β-Mercaptoethanol (BDH Chemicals, Poole, England) and soybean lipids (Boehringer Mannheim) were obtained in soluble form. Linoleic acid (5353, Merck, Darmstadt, West Germany) was first dissolved in 96% ethanol. After evaporation of the ethanol, it was dissolved in RPMI 1640 with 1% BSA followed by three 10-second pulses of ultrasonification. All additives were further diluted in RPMI 1640 with penicillin, streptomycin, and fungizone. Microculture plates with 20 μL of medium additives in triplicate were stored at −20°C.

Aliquots (80 μL) of a cell suspension containing ITS were placed into the microculture plates containing 20 μL of medium additives. A cell suspension lacking ITS was added to wells filled with insulin,

transferrin, selenium, or ITS. After 4 days of incubation, LCS was determined by measuring MTT reduction as described above. Because pyruvate and BSA have significant OD measurements, the data were corrected for this.

The influence of ITS on the proliferation rate of cells was studied. Flow cytometry was performed as described before.[18] Briefly, percentages of cells in different phases of the cell cycle were calculated by a planimetrical method[19] from DNA histograms obtained with the Phywe 11 flow cytometer after 4 days' culture in medium with or without ITS. 2×10^6 cells fixed in 70% alcohol were treated with 0.5 mg/ml RNAse (Boehringer, Mannheim, West Germany) in Tris buffer for 30 minutes and with pepsine for 20 minutes at 37°C. The cells were stained with ethidium bromide (Hoechst) and filtrated before measuring. From cells cultured 1 or 2 days in medium with or without ITS for chromosomal analysis, the number of metaphases per 3,000 cells in triplicate was counted.

Two methods to increase OD were studied: the addition of sodium succinate (BDH Chemicals, Poole, England)[20] and the incubation of cells for one more hour after addition of isopropanol.[21]

Statistics. The Wilcoxon matched-pairs signed-ranks and Mann-Whitney U tests were used for two-tailed testing at a level of significance of .05.

RESULTS

Culture medium and test conditions. The addition of fungizone and gentamycin to the culture medium was necessary in order to prevent contamination in culturing the cryopreserved samples. These two antibiotics were not toxic to the ALL cells: mean cell survival expressed as percentage of controls was 99% (range 85% to 109%; n = 6) with fungizone and 97% (86% to 103%) with gentamycin.

In two of nine samples the control cell viability was too low to assess any influence of medium additives. Results of the seven successful cases (Fig 1A and B) show that ITS increased cell survival by 25% to 62%. This increase was only significant at the intermediate concentration ($P < .01$). Testing the components separately, transferrin and selenite did not improve cell survival but 5 μg/mL insulin increased survival by 39% ($P < .05$). Although it seemed that the lowest concentration of insulin had more effect than both higher concentrations, this difference was not significant. The addition of BSA to RPMI-FCS-ITS resulted in a further 15% to 18% increase in cell survival, which was significant at

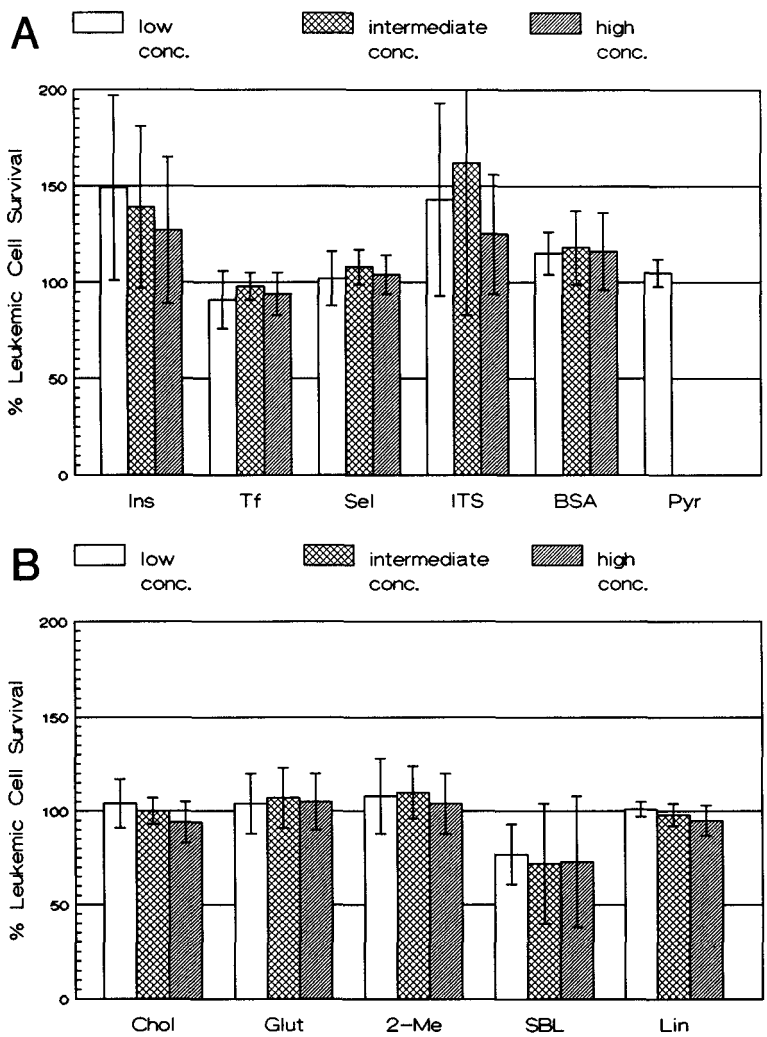

Fig 1. (A and B) Influence of medium additives upon leukemic cell survival (mean ± SD) presented as percentages of untreated control cells. Three concentrations of each additive were tested. The concentrations of ITS, a mixture of insulin (Ins), transferrin (Tf) and selenite (Sel), were mixtures of the three low, intermediate, and high concentrations of Ins (1, 5, and 10 μg/mL), Tf (1, 5, and 10 μg/mL), and Sel (1, 5, and 10 ng/mL). ITS, Ins, Tf, and Sel were added to RPMI-FCS; the other additives to RPMI-FCS-ITS. The concentrations of the other additives tested were 1, 2, and 5 mg/mL bovine serum albumin (BSA); 1, 5, and 10 μg/mL cholesterol (Chol); 5, 10, and 20 μg/mL glutathion (Glut); 25, 50, and 100 μmol/L β-mercapto-ethanol (2-ME); 10, 50, and 100 μg/mL soybean lipids (SBL); 1, 2, and 5 μg/mL linoleic acid (Lin); and 110 μg/mL pyruvate (Pyr).

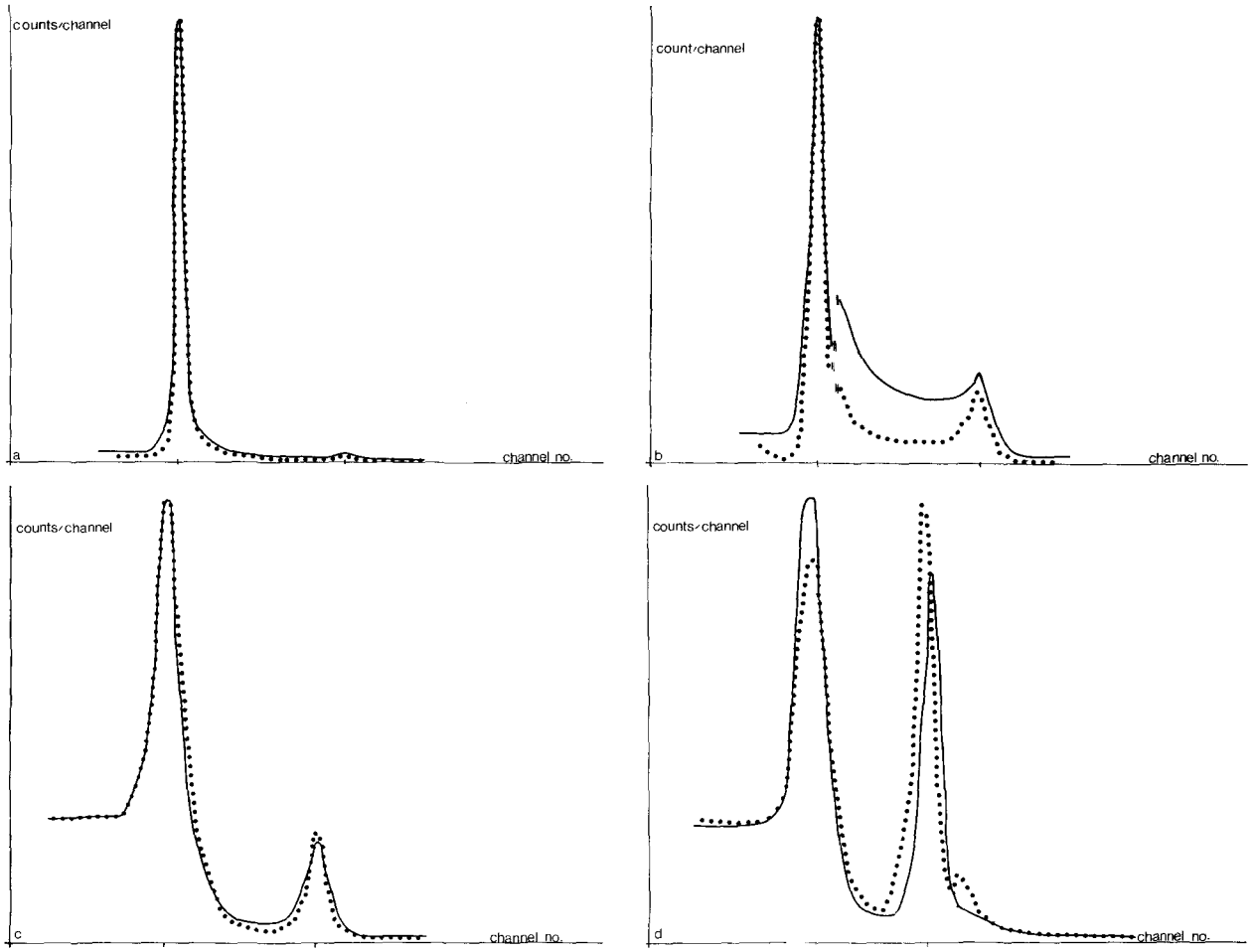

Fig 2. Distribution of cells in the different phases of the cell cycle for four ALL samples (a through d) cultured with (....) or without (——) ITS. The first peak represents the G_1 phase, the second peak the G_2 + M phase, and the area between these the S phase. In (b) different scales are used for the G_1 peak versus the S and G_2 + M phase.

1 mg/mL BSA ($P < .05$). No other additives influenced cell survival, except soy bean lipids, which gave a significant, sometimes strong decrease in ALL cell viability.

In four of seven cases the distribution of cells in phases of the cell cycle could be measured successfully. The percentages of cells in S-phase and G_2 + M phase were not increased by ITS (Fig 2). The percentage of metaphases counted in five ALL cases was not altered by ITS. Adding ITS to the medium did not appear to increase the likelihood of detecting an abnormal clone for cytogenetic analysis (Dr R. Slater, personal communication).

Directly after thawing, the MTT reduction by 1.6×10^5 ALL cells, which is the number of cells in a well when using a suspension of 2×10^6 cells/mL, resulted in an OD of 0.249 (±0.042; n = 13). Addition of 5 μmol/L succinate significantly increased MTT reduction by only 7% ± 6% (mean ± SD; $P < .05$; n = 9). Higher concentrations (10, 20, and 40 μmol/L) did not significantly influence OD (percentages of control OD were 103 ± 6, 101 ± 9, and 101 ± 6, respectively). Incubating six ALL samples at 37°C for 1 extra hour after dissolving formazan crystals with isopropanol did not increase OD values (99% ± 5%).

Drug sensitivity. The maximum number of cells inoculated per well was 1.6×10^5. The relationship between OD and cell number was linear in the tested range of 3.2 to 0.0125×10^5 cells per well ($r^2 = .976$) with OD values down to <0.025.

An assay was judged adequate for analysis when the control OD was >0.050. The MTT assay was technically successful in 54 of 87 (62%) samples. Control OD values and their coefficients of variation (CVs) of successful cases are shown in Table 1. The mean CV is 9.5%. Theoretically, the precision of measuring LC_{50} values can be compromised by background levels, leading to false high LC_{50} values in cases with a low control OD. This hypothesis was tested in the precursor B-ALL group (n = 34). No correlation was found between control OD and LC_{50} values for VCR ($r^2 = .03$), DNR ($r^2 = .07$), Ara-C ($r^2 = .01$), Must ($r^2 = .03$), Mafos ($r^2 = .04$), 6-TG ($r^2 = .10$), or L-Asp ($r^2 = .03$). For Pred, a weak positive correlation was found ($r^2 = 0.33$) instead of

the anticipated negative correlation. So background levels did not have a significant impact on the calculated LC_{50} value. In addition, the assay also has a linear relationship between cell number and OD only when low OD readings of 0.000 to 0.080 are evaluated ($r^2 = .992$). Of the OD values under 0.050, the SD is only 0.0025, which does not significantly influence the LC_{50} values of samples with a low control OD (ie, between 0.050 and 0.100). Together, these findings implicate that the assay is accurate in the lower OD range.

Dose-response curves were obtained for 6-MP, 6-TG, Pred, DNR, VCR, Ara-C, L-Asp, Mafos, and Must. In only a few patients a cytotoxic effect of MTX was observed. The LC_{50} values of ALL patients are expressed in Fig 3A through H and Table 2. Comparing leukemic cells from untreated patients with leukemic cells from relapsed patients, the latter were significantly more in vitro resistant to 6-TG ($P = .006$), Ara-C ($P = .002$), Pred ($P = .01$), Mafos ($P = .002$), and Must ($P = .005$), but not to VCR, DNR, or L-Asp. Samples from relapsed patients differ with respect to number and kind of drugs for which they show high LC_{50} values and also with respect to the degree of in vitro resistance. Also, large excursions in LC_{50} values among samples are detected in newly diagnosed patients, in many cases falling within the range of the relapsing patients. Cells from the B-ALL patient showed relatively high LC_{50} values for VCR and DNR but low for Mafos and Must.

Illustrative for the wide range of LC_{50} values are the Pred data. The patients can be divided roughly into two groups, one with LC_{50} values <1 μg/mL and the other with LC_{50} values >100 μg/mL. In one AUL and two pro-B ALL cases (patients 3, 6, and 7) Pred increased LCS, a reproducible phenomenon seen in BM as well as in PBL. BM and PBL from case 7 also showed a dose-dependent increase in cell survival upon VCR treatment. Cells from this patient showed

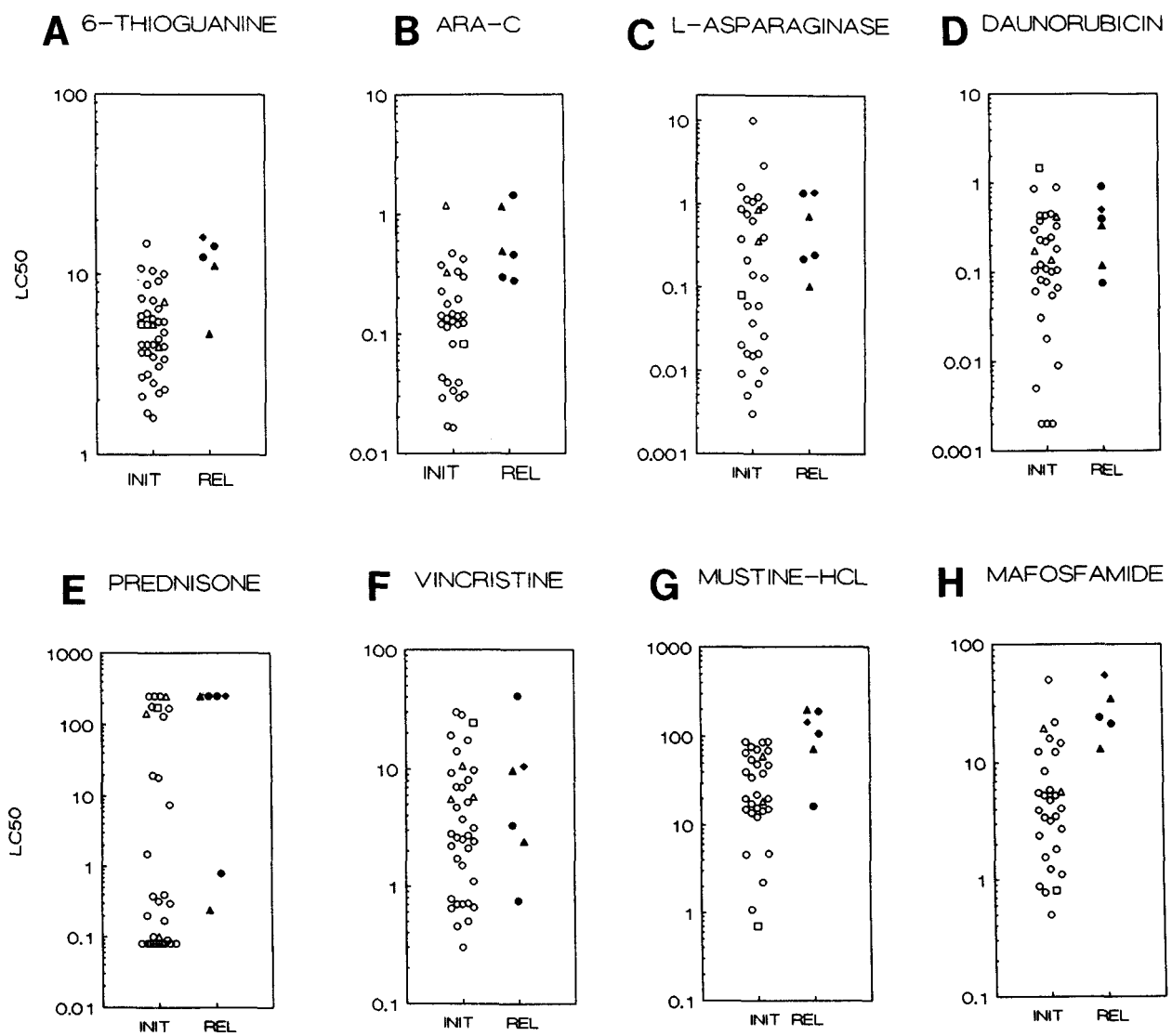

Fig 3. (A through H) LC_{50} values in μg/mL except for L-Asp (IU/mL) from cells from untreated patients at initial diagnosis (INIT, open symbols) and from relapsed patients (REL, closed symbols), obtained with the in vitro MTT assay. (○) Initial precursor B; (□) initial B; (△) initial T; (▲) relapsed T; (●) relapsed precursor B; (♦) relapsed mixed ALL/AML.

Table 2. LC_{50} Values of Newly Diagnosed and Relapsed ALL Patients

Drug	Mean ± SD		Median (Range)	
	Initial	Relapsed	Initial	Relapsed
6-TG	5.3 ± 2.9	11.8 ± 3.9	4.6 (1.6–14.9)	12.5 (4.7–16.1)
AraC	0.186 ± 0.220	0.687 ± 0.446	0.127 (0.016–1.188)	0.475 (0.278–1.440)
L-Asp	0.772 ± 1.798	0.668 ± 0.526	0.141 (0.003–10.00)	0.483 (0.103–1.371)
DNR	0.247 ± 0.308	0.388 ± 0.277	0.122 (0.002–1.470)	0.363 (0.075–0.910)
Mafos	7.58 ± 9.84	29.5 ± 14.2	4.1 (0.5–50)	24.0 (13.1–54.4)
Must	35.41 ± 27.91	120.8 ± 64.2	21.1 (0.7–87.0)	125.0 (16.2–198.4)
Pred	57.6 ± 92.2	166.8 ± 117.6	0.31 (0.08–250)	250 (0.24–250)
VCR	6.48 ± 7.77	11.28 ± 13.78	2.80 (0.30–29.81)	5.50 (0.75–41.00)

LC_{50} values of cells from newly diagnosed, initial patients and relapsed patients given in μg/mL; values for L-Asp given in IU/mL. Differences between these two groups were tested with the Mann-Whitney U test. In some cases the LC_{50} is higher than the highest concentration of a drug tested or lower than the lowest concentration tested. In the calculation of mean ±SD values, the highest or lowest concentration is taken as LC_{50} for these cases.

an unusual culture pattern characterized by colony-forming units within 4 days.

The influence of cell concentration on the MTT assay was assessed by testing 1×10^6 and 2×10^6 cells/mL simultaneously from four samples (Fig 4). LCS data correlated well ($r^2 = .760$; n = 60). The absolute differences in LCS values for both cell concentrations (mean 0.7%; n = 60) were not significant ($P = .572$). The best fit line was close to x = y and dose-response curves (not shown) were similar for both cell concentrations.

DISCUSSION

Methodological aspects. Clonogenic assays are often considered to be most suitable for determining in vitro drug sensitivity, but in cases of ALL cells they pose practical problems (they are laborious and time consuming, and have low cloning efficiency) that make testing on a large scale a problem.[22] Besides this, it has been argued that "neither theoretical concepts, direct experimental data, nor clinical correlations supported the alleged superiority of clonogenic assays."[2] These arguments were supported by the fact that the nonclonogenic DiSC assay showed favorable correlations between in vitro sensitivity and clinical response to chemotherapy; this was especially shown in lymphatic malignancies. The DiSC assay is therefore considered to be the best assay available for testing the leukemia/lymphoma population.[2-4] The disadvantages of the DiSC assay being laborious and subject to observer error can be overcome with the MTT assay. We adapted the MTT assay to test lymphoblasts of children with ALL and showed that the results were comparable with those obtained with the DiSC assay.[12,13] This was confirmed by others using fresh chronic lymphocytic leukemia (CLL) cells.[23]

Compared with cell lines, myeloid leukemia, and CLL cells, ALL cells show a low control cell viability.[13,24] After 4 days of culturing, the survival of ALL cells is about 40%.[24] However, drug sensitivity is still determined on a large fraction of the total number of cells. For comparison, in a clonogenic assay the cloning efficiency of ALL cells is only 0.06% in no more than half of the samples.[25] Although in the present study all 13 ALL samples tested directly after thawing were able to reduce MTT, the amount of reduction per cell is low compared with myeloid cells.[13,23] Thus, low OD readings at day 4 are a result of both low reduction of MTT

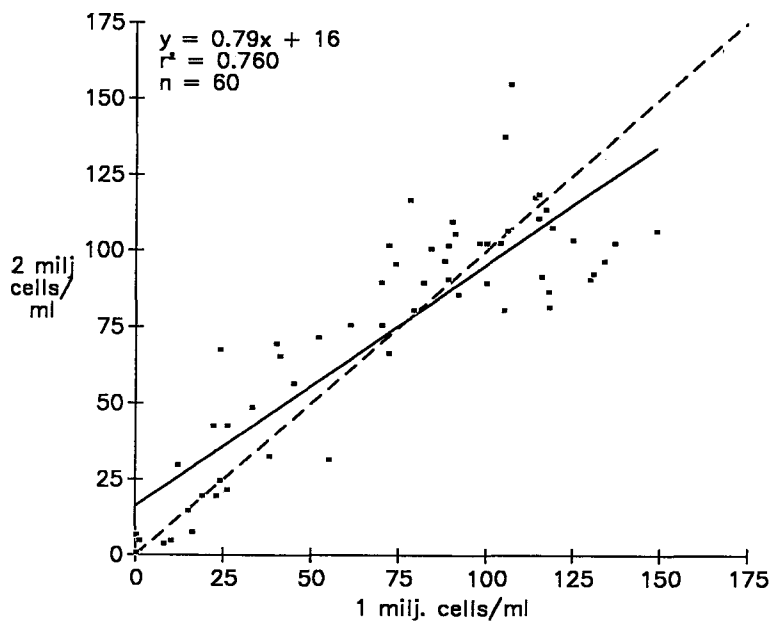

Fig 4. Comparison of drug sensitivity of cell concentrations of 1 and 2 × 10^6 cells/mL. Each point represents a paired leukemic cell survival (LCS) value (%). (——) Line of best fit; (------) line of x = y.

and control cell death. Addition of albumin, insulin, and transferrin to serum-free medium is essential for survival of human lymphoblasts.[26] In the present study, we confirmed the finding of Bird et al[24] that addition of ITS to serum-containing medium improved ALL cell survival. This effect appeared to be due to insulin only. ITS did not induce ALL cells to proliferate as shown by the flowcytometric and cytogenetic data. Adding BSA to RPMI-FCS-ITS further improved cell survival with about 15%. Other additives did not improve ALL cell viability. Soybean lipids were even toxic.

Sodium succinate enhanced reduction of MTT to formazan by sarcoma cells.[20] In the present study 5 μmol/L succinate increased MTT reduction by ALL cells with only 7%, which is of no practical importance. Also, higher concentrations of succinate did not increase the MTT reduction. The finding that higher OD values were obtained by incubating cells for 1 hour after addition of isopropanol[21] could not be confirmed by us when using ALL cells. However, measuring the OD immediately after solubilizing formazan crystals with isopropanol might result in lower OD values because it takes about 10 minutes before the OD reaches its maximum value. Some authors observed precipitation of protein in the culture medium when using acid isopropanol. We and others[21,27] did not observe this problem. Protein precipitation was seen only once when we used isopropanol, which was acidified directly before use.

The results of in vitro chemosensitivity testing of different cell concentrations (1 and 2 × 10^6 cells/mL) did not differ. Others found significant differences when comparing leukemic cell concentrations that differed by fivefold.[28]

In vitro drug resistance. The knowledge about drug resistance in childhood cases of ALL is limited. Currently, a few reports have been published on the use of the MTT assay for measuring in vitro drug sensitivity of leukemic cells obtained from patients.[12,13,21,23,27,29,30] Except for one Japanese report[29] and our own articles in which a few ALL samples were studied, these reports described results primarily for samples from CLL and acute myelogenous leukemia samples. In this study we presented the first data of a large group of children with ALL. Based on this experience, we now use the in vitro concentrations of drugs given in Table 3. A problem recently recognized with use of 6-MP is a decreasing cytotoxic effect with time, which is probably caused by the instability of the drug. Therefore, the tabulated concentrations for this drug are not yet fixed.

Table 3. Concentration of Drugs for In Vitro Drug Sensitivity Testing of ALL Cells Using the MTT Assay

Drug	Concentration Range
6-MP	15.6–500 μg/mL
6-TG	1.56–50 μg/mL
Methotrexate	0.005–500 μg/mL
L-Asp	0.0032–10 U/mL
DNR	0.002–2 μg/mL
VCR	0.049–50 μg/mL
Pred	0.08–250 μg/mL
Ara-C	0.0024–2.5 μg/mL
Mustine	0.16–500 μg/mL
Mafosfamide	0.098–100 μg/mL

We could detect a cytotoxic effect of methotrexate in only a few cases. A possible reason for this is that high concentrations of nucleosides originating from dead cells lead to increased use of salvage pathways for purine and pyrimidine metabolism by the surviving ALL cells, which are therefore rescued from the cytotoxic effect of MTX.[31,32] This results in a lack of in vitro cell kill when testing cells from patients with ALL. When leukemic cell lines (with low numbers of cells inoculated and low numbers of cells that are dying spontaneously) were tested in our system, the anticipated dose-response curves of MTX were found (unpublished data).

The cytotoxic effect of Pred was not always dose dependent. The fact that the LC_{50} data for this drug roughly divide the patients into one sensitive and one resistant group was also found by Galili.[33] It might reflect the in vivo resistance of some ALL patients to monotherapy with this drug.[34,35] In some patients, in vitro Pred treatment even caused an increased cell survival. This has been observed by others, too.[36,37] A nationwide prospective study to correlate in vitro Pred sensitivity with in vivo response to Pred monotherapy in childhood ALL is now in progress in collaboration with the Dutch Childhood Leukemia Study Group.

The fact that dose-response curves were found for L-Asp is remarkable because this drug acts by depleting the medium of asparagine. According to Asselin et al,[38] the medium would eventually be depleted of this substrate at low concentrations of L-Asp. However, in their study of ALL cells, a dose-response effect was also found at concentrations >0.1 IU/mL, which suggests other mechanisms of L-Asp cell killing. Ohnuma et al[39] also showed a dose-dependent growth inhibitory effect of L-Asp on B-cell lines not caused by the depression of asparagine in the medium. Growth inhibition of T-cell lines was related to asparagine depletion but also was dose dependent. Thus, the dose-dependent cell kill observed in our study is probably due to a mechanism other than asparagine depletion.

The DiSC assay can be used for determining in vitro drug resistance and for studies of resistance modifyers on cells from ALL patients.[40-43] Because MTT and DiSC assays show comparable results,[13,23] the MTT assay might be a more efficient alternative for the DiSC assay. It can detect differences in vitro drug sensitivity between multiple clinically relevant forms of ALL, which could at least partly explain their different responses to chemotherapy. Two higher risk groups are relapsed patients and patients with B-ALL. B-ALL is a rare, separate clinical entity with a poor prognosis and treated with a different chemotherapy protocol in the Berlin-Frankfurt-Münster strategy. Cells from the B-ALL patient were relatively in vitro resistant to DNR, VCR, and Pred but quite sensitive to alkylating drugs in vitro. This correlates well with clinical experience with alkylating drugs in B-ALL. These in vitro findings must be expanded in a study including more B-ALL patients.

Relapsed patients show an event-free survival rate of ±20% compared with ±70% of patients at initial diagnosis. We showed that cells from relapsed patients were more in

vitro resistant to 6-TG, Ara-C, Pred, Mafos, and Must, but not to VCR, DNR, or L-Asp than cells from newly diagnosed, untreated patients. Some cells from untreated patients also showed relatively high LC_{50} values, in many instances falling within the range of relapsed patients. These findings might illustrate the fact that not only drug resistance but other factors—for instance, pharmacologic factors—influence the poor clinical prognosis after relapse. It might also be true that relapsed patients are more resistant to some drugs at the time of initial diagnosis.

Large interindividual differences exist with respect to number and kind of drugs for which relapsed patients show in vitro resistance, and with respect to the degree of resistance. This might reflect differences in the clinical pattern of resistance between patients. The fact that in vitro differences between cells from untreated and relapsed patients were not detected for all drugs could be an indication that some drugs are less frequently involved in clinical acquired resistance.

The standard way to validate an in vitro drug sensitivity assay is to demonstrate the predictive ability in a prospective study. In ALL patients such a study has not yet been performed, mainly because of the lack of a suitable assay. A comparison between responders and nonresponders in terms of achieving a complete remission is hard to make because the group of nonresponders is too small in children with newly diagnosed ALL. Based on retrospective clinical correlations, the DiSC assay is considered the best assay but has considerable practical disadvantages for use in large-scale studies. The more efficient MTT assay is a new alternative, making large-scale studies in ALL patients feasible. Currently we are performing two prospective nationwide studies (Dutch Cancer Society projects 89-06 and 90-05) in initial and relapsed ALL children to examine the predictive ability of the MTT assay. Very recently, two retrospective studies in adult nonlymphocytic leukemia have shown a good predictive value of the MTT assay.[27,30]

In conclusion, we believe that the development of the in vitro MTT assay highly facilitates studies of drug resistance in leukemic patients. This holds promise for the future because it might then be possible to improve treatment protocols for poor risk groups, to suggest rational modifications to standard therapy, and eventually to tailor chemotherapy on a more individual basis.

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