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Increased *in vitro* cellular drug resistance is related to poor outcome in high-risk childhood acute lymphoblastic leukaemia

Britt-Marie Frost,[1] Peter Nygren,[2] Göran Gustafsson,[3] Erik Forestier,[4] Olafur G. Jonsson,[5] Jukka Kanerva,[6] Randi Nygaard,[7] Kjeld Schmiegelow,[8] Rolf Larsson[9] and Gudmar Lönnerholm[1] On behalf of the Nordic Society for Paediatric Haematology and Oncology [1]*Department of Women's and Children's Health, University Children's Hospital,* [2]*Department of Oncology, Radiology and Clinical Immunology, University Hospital, Uppsala,* [3]*Department of Paediatric Oncology, Karolinska Institute, Stockholm,* [4]*Department of Clinical Sciences, Paediatrics, University of Umeå, Sweden,* [5]*Department of Paediatrics, Landspitali, Reykjavik, Iceland,* [6]*Hospital for Children and Adolescents, University of Helsinki, Helsinki, Finland,* [7]*Department of Paediatrics, St Olav University Hospital, Trondheim, Norway,* [8]*Paediatric Clinic II, Rigshospitalet, Copenhagen, Denmark, and* [9]*Department of Medical Sciences, Section of Pharmacology, University Hospital, Uppsala, Sweden*

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Summary. We determined the *in vitro* cellular drug resistance in 370 children with newly diagnosed acute lymphoblastic leukaemia (ALL). The resistance to each of 10 drugs was measured by the fluorometric microculture cytotoxicity assay (FMCA) and was related to clinical outcome. The median follow-up time was 41 months. Risk-group stratified analyses indicated that *in vitro* resistance to dexamethasone, doxorubicin and amsacrine were each significantly related to the probability of disease-free survival. In the high-risk (HR) group, increased *in vitro* resistance to dexamethasone ($P = 0{\cdot}014$), etoposide ($P = 0{\cdot}025$) and doxorubicin ($P = 0{\cdot}05$) was associated with a worse clinical outcome. Combining the results for these drugs provided a drug resistance score with an independent prognostic significance superior to that of any other factor studied, with a relative risk of relapse in the most resistant group 9·8 times that in the most sensitive group ($P = 0{\cdot}007$). The results in the intermediate-risk (IR) and standard-risk (SR) groups were less clear cut. In conclusion, our data indicate that *in vitro* testing of cellular drug resistance can be used to predict the clinical outcome in HR ALL, while the final evaluation of the results in IR and SR patients must await longer follow-up.

Keywords: acute lymphoblastic leukaemia, childhood, drug resistance, *in vitro* assay, cytotoxicity.

The prognosis in childhood acute lymphoblastic leukaemia (ALL) has gradually improved over the past decades by virtue of improved and intensified combination chemotherapy (Gustafsson *et al,* 2000; Schrappe *et al,* 2000). However, 20–25% of patients still die of resistant or relapsed disease, indicating a need for more effective treatment in this subgroup. There is also growing concern that some patients might be overtreated, with an increased risk of unnecessary side-effects. Hence, there is an obvious need for methods that will allow more individualized therapy (Pui & Evans, 2001).

The degree of cellular cytotoxic drug resistance might be one of the important determinants of the outcome of treatment. Several methods for testing cellular drug resistance *in vitro* have been described, each with its advantages and disadvantages. Clonogenic assays and the differential staining cytotoxicity (DiSC) assay have shown good predictive capacity, but are labour intensive and time consuming (Hamburger & Salmon, 1977; Weisenthal *et al*, 1983; Weisenthal & Lippman, 1985). Furthermore, ALL cells clone to a lower extent than most other tumour cells, which makes the technical success rate in clonogenic assays in ALL unacceptably low (von Hoff, 1988; Pieters *et al*, 1989). In childhood leukaemias, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay – a total cell kill assay reported to be objective and non-laborious (Kaspers *et al*, 1997) – has been used hitherto in most studies. We have previously described the sensitive and reproducible semiautomated fluorometric microculture cytotoxicity assay (FMCA), and documented its feasibility and validity for *in vitro* drug resistance testing of tumour

Correspondence: Britt-Marie Frost, MD, Department of Women's and Children's Health, University Children's Hospital, SE-751 85 Uppsala, Sweden. E-mail: Britt-Marie.Frost@kbh.uu.se

cells from cell lines and patients (Larsson *et al*, 1992; Larsson & Nygren, 1993), including samples from patients with ALL (Nygren *et al*, 1992).

Significant correlations between the results of *in vitro* drug resistance testing and the clinical outcome in childhood ALL have been found in a number of studies, most of which were retrospective or reported relatively small numbers of patients (Asselin *et al*, 1989; Hongo *et al*, 1990; Nygren *et al*, 1992; Pieters *et al*, 1994). Kaspers *et al* (1997) published data on *in vitro* cellular resistance in 152 children with newly diagnosed ALL, studied by the MTT method. Hongo *et al* (1997, 1999) reported results using the same method in samples from 209 children with ALL. Although these studies comprised a considerable number of patients, there are uncertainties as to whether or not they were fully representative of the patient population from which they were recruited.

We report the results of *in vitro* testing by the FMCA in 370 children with newly diagnosed ALL, a group representative of the whole population of children with ALL in the Nordic countries during the study period, as shown by similarities in sex, age and white blood cell count (WBC) at diagnosis, immunophenotype, and probability of disease-free survival (p-DFS). The purpose was to measure the *in vitro* cellular resistance to each of 10 drugs at diagnosis, and study the correlation to established risk factors and the clinical outcome.

MATERIALS AND METHODS

Patients and patients' samples. Children (aged 1–18 years) with non-B ALL, diagnosed between 1992 and December 2000, were eligible for the study. All Swedish centres for paediatric oncology participated and provided samples from 347 patients. After 1995, an increasing number of centres in the other Nordic countries also provided samples: 86 from Norway, 35 from Denmark, 32 from Finland and six from Iceland. Thus in total, bone marrow and/or peripheral blood samples from 506 patients were received for analysis. A total of 1589 children were diagnosed with ALL in the Nordic countries during the study period.

The diagnosis of ALL was established at a paediatric oncology centre by analyses of bone marrow aspirates, including morphology, immunophenotype and cytogenetics of the leukaemic cells.

The samples were collected in heparinized glass tubes, kept at room temperature and sent by mail or through international express delivery companies. As a rule, they reached the laboratory for processing within 24–36 h. Most of the samples were analysed upon receipt (89% of the successful assays), but for practical reasons some were cryopreserved in culture medium, containing 10% dimethyl sulphoxide (DMSO) and 50% fetal calf serum (FCS), by initial freezing for 24 h at −70°C followed by storage in liquid nitrogen. The cells were later thawed and analysed. Previous studies had shown that cryopreservation did not affect the *in vitro* sensitivity to standard drugs (Pieters *et al*, 1988; Kaspers *et al*, 1991; Nygren *et al*, 1992). Bone marrow and peripheral blood samples were evaluated together, because they do not differ in drug resistance (Larsson *et al*, 1992; Nygren *et al*, 1992; Kaspers *et al*, 1997).

Leukaemic cells were prepared by 1·077 g/ml Ficoll–Isopaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation. Viability was determined by the Trypan-blue exclusion test. The median viability was 95%, and FMCA was performed only when the viability was ≥ 70%. An independent haematologist estimated the proportion of leukaemic cells on May–Grunwald–Giemsa-stained cytospin preparations, using light microscopy. The median proportion of lymphoblasts after separation was 90%, and FMCA was performed only when this proportion was ≥ 70%.

FMCA procedure. All samples were tested in the same laboratory in Uppsala.

FMCA is based on measurement of fluorescence generated from the hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes, and has been described in detail previously (Larsson *et al*, 1992; Larsson & Nygren, 1993; Nygren *et al*, 1994; Fridborg *et al*, 1999). Leukaemic cells (10^5) in 180 μl culture medium were seeded per well in 96-well microtiter plates prepared in advance with the different drugs to be tested. The culture plates were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 for 72-h continuous drug exposure. The plates were then centrifuged (200 ***g***, 5 min) and the medium removed by automatic pipetting. After one wash with phosphate-buffered saline (PBS), 200 μl/well of PBS, containing FDA (10 μg/ml), was added. Subsequently, the plates were incubated for 1 h at 37°C and the fluorescence was then read by a scanning fluorometer (Fluoroscan 2; Labsystems OY, Helsinki, Finland). Drugs were tested in triplicate. Six wells without drugs served as controls and six wells containing culture medium only served as blanks. Quality criteria for a technically successful assay included a proportion of leukaemic cells of ≥ 70% in control wells after 72 h of incubation, a fluorescence signal in the control wells of > 5 times the mean blank value and a mean coefficient of variation (CV) in the control wells of < 30%. The results are presented as a survival index (SI), defined as:

$$\text{fluorescence in test wells}/(\text{fluorescence in control wells} - \text{fluorescence in blank wells}) \times 100.$$

Thus, a low numerical value indicates high sensitivity to the cytotoxic effect of the drug.

The investigators were blinded to the clinical data when the tests were performed and evaluated.

Reagents and drugs. FDA (Sigma, St Louis, MO, USA) was dissolved in DMSO (Sigma) and kept frozen (−20°C) as a stock solution (10 mg/ml) protected from light. Culture medium [Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma)] supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Cramlington, UK), 2 mmol/l glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin (HyClone) was used throughout.

Table I. RR of relapse in children with newly diagnosed ALL with intermediately sensitive (score 2) or resistant cells (score 3) as compared with patients with sensitive cells (score 1).

Drug	Concentration	*n*	Score	RR	95% CI	*P*
Dexamethasone	1·4 μg/ml	277				
			2	2·5	1·2–5·5	0·017
			3	2·3	1·1–4·9	0·037
Etoposide	5 μg/ml	309				
			2	0·8	0·4–1·7	0·58
			3	1·7	0·8–3·2	0·12
Doxorubicin	0·5 μg/ml	287				
			2	1·2	0·6–2·5	0·56
			3	2·2	1·0–4·5	0·039
Amsacrine	1 μg/ml	294				
			2	0·8	0·4–1·6	0·47
			3	2·1	1·1–4·1	0·027
Prednisolone	50 μg/ml	309				
			2	2·1	1·0–4·3	0·047
			3	1·9	0·9–4·1	0·091
Cytarabine	0·5 μg/ml	306				
			2	1·4	0·7–2·6	0·37
			3	1·1	0·6–2·4	0·71
Vincristine	0·5 μg/ml	318				
			2	1·3	0·7–2·6	0·38
			3	1·1	0·6–2·3	0·75
6-thioguanine	10 μg/ml	294				
			2	1·5	0·8–3·0	0·23
			3	1·0	0·5–2·2	0·93
Asparaginase	10 U/ml	269				
			2	1·3	0·6–2·7	0·53
			3	1·3	0·6–2·6	0·52
4-HC	2 μg/ml	152				
			2	1·9	0·7–5·6	0·23
			3	2·6	0·9–7·2	0·075

Cox regression analysis with stratification into risk groups. For each drug, patients were divided into three equally large groups with sensitive (score 1), intermediately sensitive (score 2) and resistant cells (score 3). Samples with score 1 form the reference group.

RR, relative risk; CI, confidence interval; 4-HC, 4-hydroperoxy-cyclophosphamide.

The cytotoxic drugs were obtained from commercial sources and tested at the concentrations shown in Table I. The active metabolite, 4-hydroperoxy-cyclophosphamide (4-HC), was used instead of cyclophosphamide, a prodrug that is inactive *in vitro*. Experimental plates were prepared with 20 μl/well of drug solution at 10 × the desired final concentration. The plates were stored at −70°C until further use.

The drugs tested were used at empirically derived cut-off concentrations, chosen to produce a large scatter of SI values among the samples. These concentrations were adopted from previous studies of leukaemic cells (Nygren *et al*, 1992).

Treatment. All patients in the study were treated in accordance with the Nordic Society of Paediatric Haematology and Oncology (NOPHO) ALL-92 protocol, with stratification into one of three risk groups (Table II) (Gustafsson *et al*, 1998). Induction treatment included prednisolone 60 mg/m^2 p.o. daily, vincristine 2 mg/m^2 i.v. × 6, doxorubicin 40 mg/m^2 i.v. × 3, asparaginase 30 000 U/m^2 i.m. × 10 and methotrexate intrathecally × 4 in all risk groups. High-risk (HR) patients received an extra dose of doxorubicin on treatment d 8. After induction treatment (50 d), further chemotherapy was adapted according to risk group. In standard-risk (SR) patients the total duration of treatment was 2·5 years, and in intermediate-risk (IR) and HR patients it was 2 years (Gustafsson *et al*, 2000).

Clinical outcome. Assessment of the treatment results was based on bone marrow morphological analyses, peripheral blood tests and cerebrospinal fluid examinations. The results were evaluated from annual reports submitted from the treating clinicians to the NOPHO registry at the Childhood Cancer Research Unit in Stockholm. The date of the last follow-up was 31 December 2001.

Complete remission (CR) was defined as less than 5% leukaemic blasts in a representative bone marrow sample and no manifestation of leukaemia elsewhere.

Relapse was defined as more than 5% blast cells in bone marrow and/or manifestation of leukaemia elsewhere.

Table II. Criteria for risk grouping of children with non-B ALL, according to the NOPHO ALL-92 protocol.

Risk group	Age (years)	Criteria
Standard	2 < 10	WBC < 10×10^9/l
		No high-risk criteria
Intermediate	2 < 10	WBC 10 < 50×10^9/l or
	1 < 2 or ≥ 10	WBC < 50×10^9/l
		No high-risk criteria
High	≥ 1	and at least one of the following criteria:
		WBC ≥ 50×10^9/l
		Mediastinal mass
		CNS or testicular involvement
		t(9;22), $22q^-$, t(4;11)
		T-cell leukaemia
		Slow response: > 25% leukaemic cells d 15 or > 5% leukaemic cells d 29

WBC indicates white blood cell count at diagnosis. CNS, central nervous system.

Resistant disease (RD) was failure to achieve CR within 50 d of induction therapy, and was considered an event on d 0. Induction death was death before completion of induction therapy (50 d). Disease-free survival (DFS) was defined as the time from diagnosis to a leukaemia-related event, relapse or RD. For estimation of DFS, induction deaths were censored at d 0 and deaths in continuous complete remission (CCR) were censored at the time of occurrence. Patients in CCR were censored at the time of the latest follow-up.

Statistical analysis. Non-parametric methods were used throughout. Differences in distribution of variables were tested with the Mann–Whitney *U*-test, Kruskal–Wallis *H*-test or the chi-squared test. The Spearman's correlation coefficient was used to examine relationships between continuous variables. Curves illustrating the probability of DFS were calculated by the Kaplan–Meier method. The log-rank test was used to compare survival curves. Statistical comparisons of outcome were conducted by simple (univariate) and multiple Cox proportional hazard regression analysis, with stratification for risk group when the whole patient material was analysed. The model for multiple analysis included age, WBC, immunophenotype and *in vitro* cellular drug resistance as covariates.

The STATISTICAL PACKAGE FOR THE SOCIAL SCIENCES (SPSS), version 10·0, software was used for the calculations. All analyses were two tailed and the level of statistical significance was set at $P < 0{\cdot}05$.

Local ethics committees approved the study.

RESULTS

Testing of cellular resistance by FMCA was accomplished successfully in 370 of the 506 samples reaching the laboratory. Reasons for failure were: total number of cells too small to test any drug ($n = 39$), too low proportion (< 70%) of lymphoblasts before the test ($n = 8$), too low proportion (< 70%) of lymphoblasts in control wells after 72 h of incubation ($n = 17$), too low signal in controls compared with blanks, i.e. signal-to-noise ratio < 5 ($n = 48$), and a control CV of ≥ 30% ($n = 24$). The technical success rate for samples with sufficient number of cells was 370 out of 467 (79%).

At participating centres, patients were entered consecutively in a prospective manner. However, several Nordic centres did not participate in the study, or did so only during the last years of the study period (see *Materials and Methods*), which explains the high number of children not tested. Table III shows the characteristics of the successfully tested patients, as well as those of all children treated according to the NOPHO ALL-92 protocol in the Nordic countries during the study period. Note the similarity in age, sex, WBC at diagnosis, immunophenotype, cytogenetics, allocation to risk group, number of events, and 3- and 5-year p-DFS, indicating that the tested patients were a representative sample of the whole patient population. Particularly important is the similarity in median WBC at diagnosis: $9{\cdot}5 \times 10^9$/l in the test group and $9{\cdot}4 \times 10^9$/l in the whole patient population. When successfully tested children were compared with children not tested or unsuccessfully tested, no statistically significant differences were found for any of the studied parameters (*P*-values in Table III).

In vitro drug resistance versus patient characteristics

Cell survival varied extensively between samples for all drugs tested. To explore this marked interindividual variation, the correlations between SI values and some basic patient characteristics were analysed. There was no significant correlation between *in vitro* drug resistance and age for any drug, either when age was treated as a continuous variable or when patients were divided into those below and above 10 years of age. Boys and girls did not differ in their *in vitro* resistance to any of the drugs tested. SI values correlated negatively to WBC at diagnosis, with $P < 0{\cdot}01$ for all drugs except 4-HC (not shown).

Table III. Characteristics of 370 children with newly diagnosed ALL, successfully tested for *in vitro* cellular drug resistance, compared with those not or unsuccessfully tested ($n = 1219$).

	Tested	Not or unsuccessfully tested	All children	*P*
No of patients	370	1219	1589	
Age, years				
median (range)	4·8 (1·1–17·4)	4·5 (1·0–17·4)	4·6 (1·0–17·4)	0·063
1–9/≥ 10 years	291/79	1006/213	1298/291	0·092
ratio	3·7/1	4·7/1	4·5/1	
Male/female	203/167	657/562	859/730	0·72
ratio M/F	1·2/1	1·2/1	1·2/1	
WBC, $\times 10^9$/l				
median	9·5 (0·5–768)	9·3 (0·3–990)	9·4 (0·3–990)	0·37
< 10	193 (52%)	632 (52%)	825 (52%)	0·99
10–50	112 (30%)	372 (30%)	484 (30%)	
> 50	65 (18%)	215 (18%)	280 (18%)	
Phenotype				0·28
B-precursor	330 (89%)	1084 (89%)	1414 (89%)	
T-cell	40 (11%)	109 (9%)	149 (9%)	
Unknown	–	24 (2%)	24 (2%)	
Modal number				
52–92 chromosomes	(46%)	(44%)	(45%)	
≤ 51 chromosomes	(54%)	(56%)	(55%)	
11q23 translocations	(2·6%)	(0·8%)	(1·9%)	
t(12;21)(p13;q22)	(25%)	(22%)	(24%)	
Ph chromosome	(2·1%)	(3·7%)	(3·0%)	
Risk groups				0·24
SR	126 (34%)	410 (34%)	536 (34%)	
IR	150 (41%)	448 (37%)	598 (38%)	
HR	94 (25%)	361 (29%)	455 (28%)	
Events in all risk groups				0·39
CCR	302 (81·6%)	961 (78·8%)	1263 (79·5%)	
Ind deaths + RD	4 + 2 (1·6%)	18 + 1 (1·6%)	22 + 3 (1·6%)	
Relapse	55 (14·9%)	210 (17·2%)	265 (16·8%)	
Deaths in CCR	7 (1·9%)	29 (2·4%)	36 (2·3%)	
Relapse in risk groups				
SR	15 (12%)	45 (11%)	60 (11%)	
IR	15 (10%)	64 (14%)	79 (13%)	
Events in HR				0·26
CCR	63 (67%)	232 (65%)	295 (65%)	
Ind deaths + RD	2 + 2 (4%)	13 + 1 (4%)	15 + 3 (4%)	
Relapse	25 (27%)	100 (27%)	125 (27%)	
Deaths in CCR	2 (2%)	15 (4%)	17 (4%)	
Follow-up time, months				
median (range)	41 (12–109)	70 (12–119)	63 (12–119)	
p-DFS ± SD at 3 year	0·86 ± 0·02	0·86 ± 0·01	0·86 ± 0·001	0·81
p-DFS ± SD at 5 year	0·79 ± 0·03	0·80 ± 0·01	0·80 ± 0·01	

P-values are from comparisons between successfully tested children ($n = 370$) and children not tested or unsuccessfully tested ($n = 1219$). The total number of children diagnosed with ALL in the Nordic countries during the study period was 1589, and samples were received from 506 of these patients. Unsuccessful tests were because of insufficient number of cells ($n = 39$) or technical failure ($n = 97$). The 39 patients from whom samples with too few cells were obtained had lower WBC at diagnosis (median 5·1; $P = 0·01$), but otherwise did not differ significantly from the successfully tested patient population (data not shown). The characteristics of the 97 patients whose tests resulted in technical failures were very similar to those of the 370 successful patients ($P > 0·05$ for all parameters; data not shown), and the same was true for the subgroup of 48 patients whose tests failed as a result of a signal-to-noise ratio < 5. Cytogenetic data were not included in these analyses because of missing information in a number of patients [modal number, t(12;21)(p13;q22)] or low counts (11q23 translocation, Ph chromosome). WBC, white blood cell count at diagnosis; SR, standard risk; IR, intermediate risk; HR, high risk; CCR, continuous complete remission; Ind deaths, death during induction therapy; RD, resistant disease; p-DFS, probability of disease-free survival.

Thus, samples from patients with high WBC were generally more drug sensitive. The correlation coefficients were low, however, ranging between −0·1 and −0·3.

In vitro drug resistance versus clinical outcome

To enable comparison with previously published data (Kaspers *et al*, 1997), the patients were classified into three equally large groups, as sensitive (33% lowest SI values), intermediately sensitive (33% intermediate SI values), or resistant (33% highest SI values). Univariate Cox regression analysis showed that patients resistant to dexamethasone, etoposide, doxorubicin, amsacrine, prednisolone and 4-HC had a risk of relapse about twice that of sensitive patients (Table I). The difference was statistically significant for dexamethasone, doxorubicin and amsacrine. Multiple Cox regression analysis, including age, WBC at diagnosis and immunophenotype as covariates, generated similar results (data not shown).

In the subsequent analyses, the risk groups were treated separately. To avoid subgroups that were too small, patients within each risk group were dichotomized into those with SI values above and below the median for that group.

HR group. For all drugs, p-DFS Kaplan–Meier curves were plotted, as shown for dexamethasone, doxorubicin and etoposide in Fig 1. At univariate Cox regression analysis, *in vitro* data of prognostic significance (or borderline significance) for p-DFS were found for dexamethasone ($P = 0{\cdot}039$), doxorubicin ($P = 0{\cdot}056$) and etoposide ($P = 0{\cdot}055$). Of other factors studied, neither WBC, age, sex, nor immunophenotype were of prognostic significance in univariate analysis (data not shown). Multiple analysis showed the independent prognostic significance of *in vitro* resistance to dexamethasone and etoposide, with borderline significance for doxorubicin (Table IV).

We next combined the data for dexamethasone, etoposide and doxorubicin. For each of these drugs, patients with a cell survival below the median were given a score of 1, and those with cell survival above the median were given a score of 2. For each patient, a total score was calculated by adding up the individual scores, ranging from 3 (sensitive to all three drugs) to 6 (resistant to all three drugs). As shown in Fig 2, an increasing score corresponded to a gradual decrease in p-DFS. The relative risk of relapse was 9·8 times greater in patients resistant to all three drugs than in those sensitive to all three drugs ($P = 0{\cdot}007$), which was superior to any other risk factor included in the multiple Cox regression analysis (Table V).

IR group. In univariate Cox regression analysis, patients with SI values above the median for dexamethasone, etoposide, doxorubicin, amsacrine and prednisolone tended to have an increased relative risk (RR) of relapse compared with those with SI values below median (RR values 1·5, 1·5, 1·7, 2·2 and 1·6 respectively). However, the difference was not statistically different for any of the drugs.

SR group. The *in vitro* resistance was not found to be of prognostic significance for the clinical outcome for any tested drug, nor were any clear trends observed.

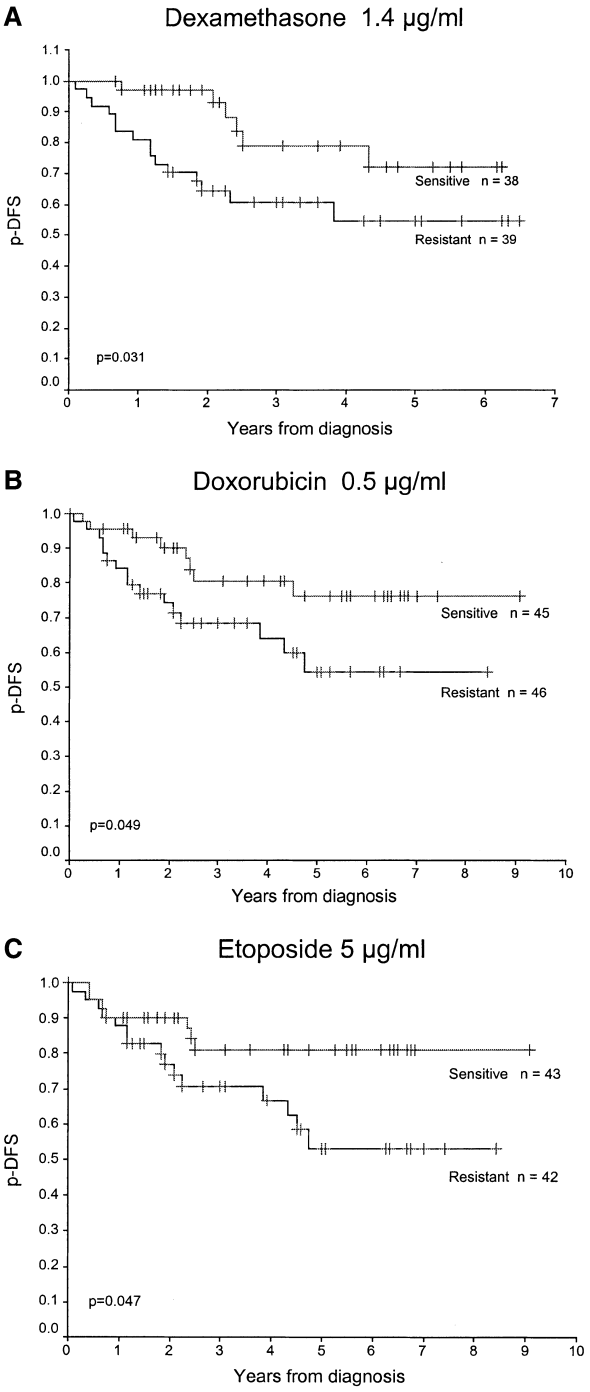

Fig 1. The relationship between *in vitro* drug resistance and p-DFS in high-risk childhood ALL: (A) dexamethasone 1·4 µg/ml, (B) doxorubicin 0·5 µg/ml and (C) etoposide 5 µg/ml. Sensitive samples had a cell survival below median, resistant samples showed a cell survival above the mean. Kaplan–Meier curves, using log-rank test.

DISCUSSION

In the present study of 370 children with newly diagnosed ALL, we found that *in vitro* resistance to dexamethasone

Table IV. RR of relapse in patients with resistant cells (cell survival above median) as compared with patients with sensitive cells (cell survival below median) in high-risk childhood ALL.

Drug	*n*	RR	95% CI	*P*
Dexamethasone	73	3·6	1·3–10	0·014
Etoposide	82	3·3	1·2–9·6	0·025
Doxorubicin	88	2·4	1·0–5·9	0·050
Amsacrine	83	2·6	1·0–6·7	0·056
Prednisolone	88	1·7	0·7–4·2	0·21
Cytarabine	85	1·7	0·7–3·8	0·23
Vincristine	88	1·5	0·6–3·7	0·34
6-thioguanine	84	1·1	0·2–2·8	0·91
Asparaginase	71	1·0	0·4–2·4	0·92
4-HC	41	1·0	0·2–4·8	0·99

Multiple Cox regression analysis including WBC at diagnosis, age and immunophenotype as covariates. Except for the drugs indicated above, none of the covariates had any prognostic significance. CI, confidence interval; 4-HC, 4-hydroperoxy-cyclophosphamide.

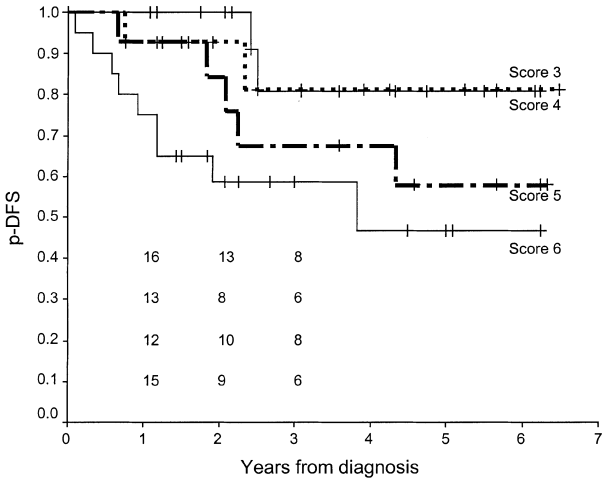

Fig 2. The relationship between *in vitro* drug resistance score, obtained by combining results for dexamethasone, doxorubicin and etoposide, and p-DFS in high-risk childhood ALL. The set of numbers located in the lower left had corner of the graph indicates the patients at risk at the different time points (1, 2 and 3 years). Log-rank test for difference between scores 3 and 6 gave a *P*-value of 0·027. There was a significant difference also when data were grouped into two categories with scores 3–4 and 5–6 ($P = 0{\cdot}016$). The broken line indicates the curve for a score of 3.

and etoposide was significantly associated with a worse clinical outcome in high-risk patients, with a borderline *P*-value for doxorubicin (Table IV). Combining the results for these drugs yielded a drug resistance score with prognostic significance superior to that of any other factor studied (karyotype not included). The findings in SR and IR patients were less clear cut. One reason for this might simply be that fewer relapses had been reported in SR and IR patients (12% and 10%, respectively, as compared with 27% in the HR group). With a small number of events, very large numbers of patients are needed to show any correlation to clinical outcome. However, there might be other causes as well. Relapses occur earlier in HR patients than in IR and (especially) SR patients, and HR ALL is sometimes characterized as 'fast disease' (Hirt *et al*, 1997). The mechanisms which allow lymphoblasts to remain dormant for long periods of time in SR and IR patients are not known in detail, but it is conceivable that *in vitro* assays are more suitable for predicting early relapses in 'fast-disease' patients than late relapses in SR and IR patients. Den Boer *et al* (2002) recently reported that patients with a resistant profile in the MTT test were at increased risk of an early event (non-response or relapse within 2·5 years of initial diagnosis) compared with patients with a sensitive or intermediate-sensitive profile. The profile did not identify patients at higher risk of late relapse. As the median follow-up of the patients of the present study is 41 months, more relapses can be expected to occur, especially among SR and IR patients, and the final analysis in these groups must await further follow-up.

Table V. RR of relapse in 64 children with newly diagnosed high-risk ALL.

Factor	RR	95% CI	*P*
Drug resistance score for etoposide + dexamethasone + doxorubicin			
Score* 4 *vs* score 3	2·1	0·3–17	0·50
Score 5 *vs* score 3	4·7	0·8–28	0·087
Score 6 *vs* score 3	9·8	1·8–52	0·007
WBC†			
50–100 *vs* < 50	4·5	1·1–19	0·038
> 100 *vs* < 50	2·4	0·5–5·7	0·24
T *vs* B phenotype	1·7	0·5–5·4	0·40
Age 1–10 *vs* > 10 years	1·7	0·5–5·7	0·42

*For each of the drugs, patients with cell survival below median (sensitive) were given a score of 1 and those with cell survival above median (resistant) were given a score of 2. For each patient, a total score was calculated by adding up the individual scores.

†White blood cell counts (WBC) were grouped into three categories: 0–50, 50–100 and $> 100 \times 10^9$/l respectively.

Multiple Cox regression analysis using a drug resistance score combining test data for dexamethasone, etoposide and doxorubicin. CI, confidence interval.

Cell survival after drug exposure correlated negatively to WBC, i.e. high WBC was correlated to high drug sensitivity *in vitro*. The low correlation coefficients indicated that WBC accounted for only a small extent of the differences between patients, but it is still a somewhat surprising finding, which has not been reported previously. We think the reason for this is that WBC reflects cell proliferation, and proliferating cells are generally more sensitive to cytotoxic agents than non-proliferating cells (Tannock, 1987). In the clinical setting, factors other than cellular drug sensitivity also come

into play, such as the rate of tumour cell regrowth, which probably explains why high WBC at diagnosis in childhood ALL strongly correlates with a worse clinical outcome (Preisler *et al*, 1984; Larsson, 1998). When *in vitro* resistance data are analysed for their correlation to clinical outcome in a group of ALL patients, WBC will act as a 'confounding factor'. This can be adjusted for by stratification into risk groups, or by analysing data for each risk group separately. As WBC is used to allocate patients to treatments of varying intensity, WBC loses much of its power as a prognostic factor within the risk groups. The same is true for age, which also failed to predict outcome within our risk groups.

Cytogenetic abnormalities of leukaemic cells have proven to be independent prognostic factors in childhood ALL (Forestier *et al,* 2000). In the present study, we have reported the cytogenetic findings in order to provide a further reason for regarding our study group as a representative sample of the whole patient population. Cytogenetic data were not included in the survival analyses, however, as a number of patients had some information missing, this would have diminished the statistical power. These data will be dealt with in a forthcoming paper.

Kaspers *et al* (1997) reported that *in vitro* resistance to each of the drugs prednisolone, asparaginase and vincristine was significantly related to the p-DFS in children with newly diagnosed ALL. Trend values were found for dexamethasone and doxorubicin, but not for cytarabine or 6-thioguanine. Amsacrine and etoposide were not included among the drugs tested. Hongo *et al* (1997) found a significantly higher 3-year event-free survival in the patients sensitive to each of the drugs prednisolone, etoposide, mitoxantrone and vincristine, with trend values for 4-HC, dexamethasone and asparaginase. Both groups reported that the combination of data for prednisolone, asparaginase and vincristine created a drug-resistance profile with prognostic significance for p-DFS (Kaspers *et al*, 1997; Hongo *et al*, 1999). To summarize, studies of childhood ALL by three independent groups, including ours, found that *in vitro* resistance to corticosteroids was of prognostic significance. The data for anthracyclines and etoposide also indicate a prognostic potential, while assays of *in vitro* resistance to 6-thioguanine and cytarabine did not yield data of prognostic significance in any of the three studies. Thus, there is good agreement between these studies for most drugs, with the exception of vincristine and asparaginase.

The asparaginase concentration used here was probably too high. As recently shown, additional mechanisms of cytotoxicity that are not relevant *in vivo* may be operative *in vitro* at the concentration of asparaginase used in the present study (Wagner *et al*, 1999). For vincristine, the reason for the divergent results is less evident. One possible reason may be differences in patient selection. As pointed out by Kaspers *et al* (1997), they tested a selected group of patients: the median WBC among patients with a successful *in vitro* test was significantly higher than in patients from whom material was not received. Hongo *et al* (1997) presented no data on the background population from which their patients were recruited, but the data that were given indicated that children with high WBC at diagnosis might have been over-represented. Finally, differences in treatment protocols might be of importance, as treatment itself is a very important prognostic factor.

As the diagnosis of the patient was unknown at analysis, the samples were tested against a panel of 10 drugs commonly used in the treatment of acute lymphoblastic and/or myeloblastic leukaemia. Two drugs not included in the NOPHO ALL-92 protocol, i.e. etoposide and amsacrine, were among the drugs for which *in vitro* resistance had prognostic significance for clinical outcome. Although the exact mechanisms are unclear at present, we hypothesize that resistance to these drugs could predict treatment failure in ALL, because cross-resistance to cytotoxic agents is common both *in vitro* and *in vivo*.

In general, *in vitro* drug resistance assays may not be applicable to all types of anticancer drugs. As the FMCA and MTT assays measure cell damage in the whole tumour cell population, consisting partly of non-dividing cells, the effect of antiproliferative drugs may not be accurately detected. Furthermore, the mechanisms of drug-induced cell death may be different *in vivo* and *in vitro*. For example, a drug may induce DNA strand lesions leading to cell-division-linked cell death *in vivo*, whereas *in vitro* death of non-dividing cells may be obtained only after more extensive DNA damage and recruitment of additional cell death pathways at supra-pharmacological drug concentrations (Kern & Weisenthal, 1990; Weisenthal & Kern, 1991; Wilbur *et al*, 1992). A drug resistance assay may still be valid provided that the mechanisms protecting the cells are similar and proportional *in vitro* and *in vivo*. This may not be true for all diagnoses and drugs, however, and may theoretically explain the lack of predictive ability for some of the drugs used in current ALL therapy (Weisenthal & Kern, 1991; Wilbur *et al*, 1992).

The main improvements in the outcome of treatment for childhood ALL over the past decades have been seen in children with non-HR leukaemia (Gustafsson *et al,* 2000). In HR ALL, the progress has been modest and, as shown here in Table III, the relapse rate in this risk group is still about 30%. In current protocols, presenting features such as WBC, immunophenotype and cytogenetics are used to identify high-risk patients, some of whom will receive extra intensive therapy, including stem cell transplantation. Response to treatment, as evaluated by bone marrow morphology or measurement of minimal residual disease (MRD), is also increasingly used for risk stratification. In the current German Cooperative ALL Study Group protocol (COALL–97), newly diagnosed children with ALL are being stratified using the MTT test for *in vitro* drug resistance (Den Boer *et al,* 2002).

Within the framework of the current NOPHO ALL 2000 protocol, we are trying to clarify whether combined monitoring of *in vitro* drug resistance at diagnosis and MRD during therapy can offer new ways to stratify the intensity of therapy. This approach has produced promising data for prednisolone (Schmiegelow *et al,* 2001).

In conclusion, we found that *in vitro* resistance to each of the drugs dexamethasone, doxorubicin and etoposide was associated with a worse clinical outcome in children with HR ALL. Combining the results for these drugs yielded a drug resistance score with an independent prognostic significance superior to that of any other factor studied. The present data support the hypothesis that *in vitro* assays are useful for predicting early relapse of ALL, while factors other than cellular drug resistance might be of greater importance for late relapses.

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