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Flow-cytometric determination of intracellular pH, esterase activity and cell volume in human leukemic cell lines following in vitro incubation with cytostatic drugs

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Abstract

A recently developed flow cytometric assay method using patient tumor cells allows the determination not only of their sensitivity to cytostatic drugs but also of biochemical and biophysical parameters after treatment, such as esterase concentration and intracellular pH of the living cells, DNA-content of the dead cells and cell volume of living and dead cells. The T-cell lines CEM, Molt4, Jurkat, the B-cell lines RPMI1788, Daudi, Raji and the promyelocytic line HL60 were incubated with: cytosine arabinoside (ara-C), L-asparaginase, daunorubicin, vincristine and prednisone for 48 h. Living cells were then stained with esterase and pH-dye 1,4-diacetoxy-2,3-dicyanobenzene (ADB) and dead cells with DNA-dye propidium-iodide (PI). The esterase concentration, an index of metabolic activity, decreased in the T-cell lines under the influence of ara-C, daunorubicin and vincristine, whereas in the B-cell lines smaller changes in esterase concentration were observed ($P<0.001$). A decrease in intracellular pH was seen in the ara-C and daunorubicin-incubated cells Molt4, CEM and HL60, whereas in the B-cell lines no significant change in intracellular pH was found. In all lines except Jurkat the cell volume of the surviving cells increased under the influence of certain drugs (primarily ara-C and daunorubicin); B-cell lines showed a greater swelling than T-cell lines ($P=0.001$).

Key words: Flow cytometry; Leukemic cell line; Intracellular hydrogen-ion concentration; Esterase; Cell-volume; pH-dye ADB; Cytostatic drug

Introduction

Many patients still die of leukemia despite recent advances in the treatment of acute leukemias in adults. Distinct risk factors have been defined for acute lymphocytic leukemia (ALL): age above 35, initial leucocyte count above $25–35 \times 10^9$/l, initial CNS-involvement, distinct immunological subtypes (e.g., B-ALL, U-ALL) [1]. In the acute myeloid leukemias (AML) risk factors are: age above 55 years, secondary leukemia after myelodysplasia. An important factor in remission induction with acute leukemias could be the sensitivity of the individual leukemic clone to cytostatic

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drugs; this can be determined by pretherapeutic cytostatic drug testing on patient cells. Three types of in vitro cytostatic drug assays have been described: (**1**) the colony-forming assay; (**2**) the short-term assay; (**3**) the xenotransplant system. The usefulness of measuring soft agar colony formation after in vitro preincubation with cytostatic drugs for the management of acute leukemias has been shown [2]. The assay is, however, time consuming and in a significant percentage of cases no tumor cell growth is obtained. The short-term assay serves to determine the inhibition of incorporation of radioactively labelled DNA-precursors into the DNA after incubation with cytostatic drugs. This assay is restricted to drugs that acutely influence DNA-metabolism; several efficacous but more slowly acting drugs, e.g., vincristine, cannot be tested [3]. Furthermore, the radiation of the labelled thymidine may influence DNA-kinetics [4]. In view of the need for a simple cytostatic drug assay, a dye-exclusion assay was published by Weisenthal [5]. A flow-cytometric system was introduced by Valet [6]. This assay screens for the reduction of viable tumor cells and inflammatory cells following in vitro cytostatic drug incubation. After an incubation period of 2–7 days cells are harvested and stained with a cocktail of two fluorescent dyes ADB and PI: (**1**) ADB diffuses through the membrane of living cells and is cleaved by non-specific esterases into the fluorescent pH-indicator dye DCH (1,4-dicyano-2,3-hydrochinone), which is trapped in the cell and accumulates; (**2**) PI stains the DNA in the dead cells. Previous work has shown the usefulness of this assay in human leukemias [7]. The aim of the present study was to investigate the changes in biochemical parameters such as esterase concentration, intracellular pH and cell volume of the surviving cells (i.e., 'drug-resistant' cells) after incubation with five different cytostatic drugs. Human leukemic cell lines were investigated as a model system.

Materials and Methods

Cell lines
The human T-cell lines Molt4, CEM and JURKAT were kindly provided by Dr Kummer, the human promyelocytic cell line HL60 by Dr Mailhammer, both from the Institut für Hämatologie der GSF, München, FRG. The human B-cell lines DAUDI, RAJI and RPMI 1788 were kind donations from Drs Johnson and Ziegler from the Institut für Immunologie, Universität München, FRG.

Exponentially growing cells were incubated for 48 h at 37 °C and 5 Vol% CO_2 in 0.9 ml RPMI 1640 (Gibco, Frankfurt, FRG) with 10% FCS (Gibco) in a 24-well titerplate (Costar, Cambridge, MA, USA) at a cell concentration of $1–4 \times 10^5$/ml. 100 µl cytostatic drug solution were pipetted into each test assay and 100 µl RPMI 1640 were pipetted into the control assay. The drugs were dissolved in RPMI1640. The cytostatic drug concentrations were (1 × therapeutic serum level): cytosine arabinoside (0.5 µg/ml) (Mack, Illertissen, FRG), L-asparaginase (0.2 U/ml) (Bayer, Leverkusen, FRG), daunorubicin (0.1 µg/ml) (Farmitalia, Freiburg, FRG), vincristine (0.01 µg/ml) (Lilly, Gießen, FRG), prednisone (10 µg/ml) (Merck, Darmstadt, FRG).

Cell-staining
The cultured cells were washed twice in the titer plates with 2 ml 10 mM Hepes buffered saline (HBS, pH 7.35) by 5 min centrifugation at $200 \times g$, followed by resuspension in 250 µl HBS. 5 µl of the dye cocktail (Cyto-P-check, Paesel,

Frankfurt, FRG) containing 1 mg/ml of the esterase and intracellular pH-indicator dye 1,4-diacetoxy-2,3-dicyanobenzene (ADB) [8] for vital cells and 2 mg/ml of the DNA dye propidium iodide (PI) for dead cells were added to each well. The cell suspension was stained for 5 min at room temperature. Previous studies have shown that incubation for 5 min at room temperature is enough to reveal stable pH measurements [8]. 10 μl of 5 μm diameter, NH_2-bearing, porous and monosized particles [9] at a concentration of 2.5×10^5/ml were added to the stained cell suspension as an internal counting, volume and fluorescence standard. The particles were prestained with 20 μg/ml 1,4-dicyano-2,3-hydrochinone (DCH, Paesel, Frankfurt, FRG) solution in HBS-buffer, pH 7.4, for 2 h at 0°C.

Flow cytometry

The stained cells were measured for 2 min in a Fluvo-Metricell flow cytometer (HEKA-Elektronik, Lambrecht/Pfalz, FRG). The cell volume was determined electrically in a hydrodynamically focused cylindrical orifice of 85 μm diameter and 110 μm length at a current of 0.23 mA. The fluorescence of the dyes was excited between 300 and 400 nm with a HBO-100 high-pressure mercury arc lamp. The blue fluorescence of the DCH was collected between 418 and 440 nm and the green fluorescence of the DCH as well as the red fluorescence of PI between 500 and 680 nm. The maximum amplitude of each signal was amplified by 2.5-decade logarithmic amplifiers. The digitized amplitudes of the three simultaneously measured signals from each cell were collected on-line in list-mode on magnetic tape. The tapes were evaluated with a VAX 11/782 (Digital Equipment Corporation, Maynard, MA, USA) by means of FORTRAN programs [10].

The list mode data were evaluated according to the following brief description: in the first step, a cloud display (Fig. 1A) was computed from the list-mode data on magnetic tape. The cloud display was then projected onto the blue-to-green fluorescence plane of the cube (Fig. 1B). A line was drawn which separated the vital and blue-stained cells from the dead cells, which were predominantly stained red by the DNA dye PI. The cube data were then reprocessed such that a cell volume versus blue-fluorescence diagram of the vital cells was obtained (Fig. 2), where the blue fluorescence was a measure of the esterase activity of the vital cells and the ratio of blue to green fluorescence a measure of the intracellular pH value. In addition, the cell volume versus red fluorescence serving as a measure of cellular DNA content of the dead cells was plotted (not shown). Surviving cells were calculated from the ratio: number of vital cells in the large cell area divided by the number of smaller calibration particles. The surviving cells in the drug assay were expressed as a % of the control assays. T- and B-cell lines were investigated once, and HL60 was investigated twice. Variance analysis was used for the statistical comparison of two groups.

Results

The reproducible evaluation of cultures in four parallel wells of the same cell suspension yielded a mean coefficient of variation of 21% concerning % surviving cells. A cell line was classified as sensitive to a cytostatic drug when the number of surviving cells was reduced to less than 10% of that of control assays. Sensitive were:

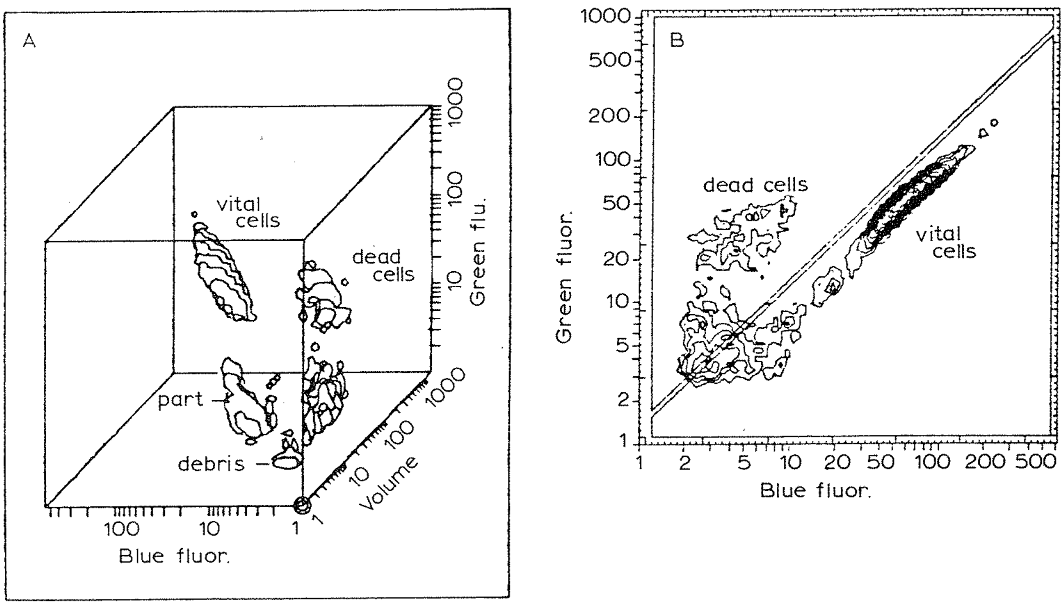

Fig. 1. (A) Cells of HL60 after a 2 day incubation without cytostatic drugs. Vital cells and calibration particles can be distinguished from dead cells. A total of 11 167 cells and particles was measured. The graph was standardized to the maximum logarithmic channel content ($N = 1196$) and contour lines plotted downwards at the 10% level. (B) Projection of the cube from (A) onto the blue vs. green fluorescence plane. The green-light channel collected the DCH green fluorescence but also the red fluorescence of the DNA-dye PI. The intracellular cleavage of ADB to DCH, followed by DCH accumulation, takes place only in living cells. A separation line is drawn which separates surviving and dead cells for individual analysis. Plotted is the measurment of 8896 cells and particles. The maximum channel content is 693 cells and contour lines are plotted downwards at the 10% level.

HL60 (ara-C, L-asparaginase, daunorubicin, vincristine), Raji (Fig. 3) (vincristine), Jurkat (daunorubicin) and CEM (ara-C, L-asparaginase, daunorubicin). Resistant were: RPMI1788, Daudi and Molt4.

Esterase concentration

When the reprocessed list-mode data of the surviving cells were projected onto the blue vs. green fluorescence plane of the three-parameter cube (Fig. 1A) the esterase activity could be estimated (Fig. 1B). The ratio, esterase activity/cell volume was calculated for each measurement and, in order to obtain a measure independent of the cell volume, was referred to as esterase concentration. T-cell lines showed a significant reduction in ara-C, daunorubicin and vincristine treated cultures, whereas in B-cell lines smaller changes in esterase-concentration were observed (Table 1). The difference between T- and B-cell lines was significant ($P < 0.001$). The line HL60 showed no significant change in esterase concentration (Table 1).

Intracellular pH

The ratio blue to green fluorescence is a measure of intracellular pH (pH_i) [8, 11]. The suitability and the reproducibility of ADB to measure intracellular pH has been investigated previously [8, 11]. ara-C, daunorubicin and vincristine caused a decrease in pH_i in several cell lines (Table 2). The difference in terms of pH_i between T- and B-cell lines was not significant when the effect of incubation with all five drugs was

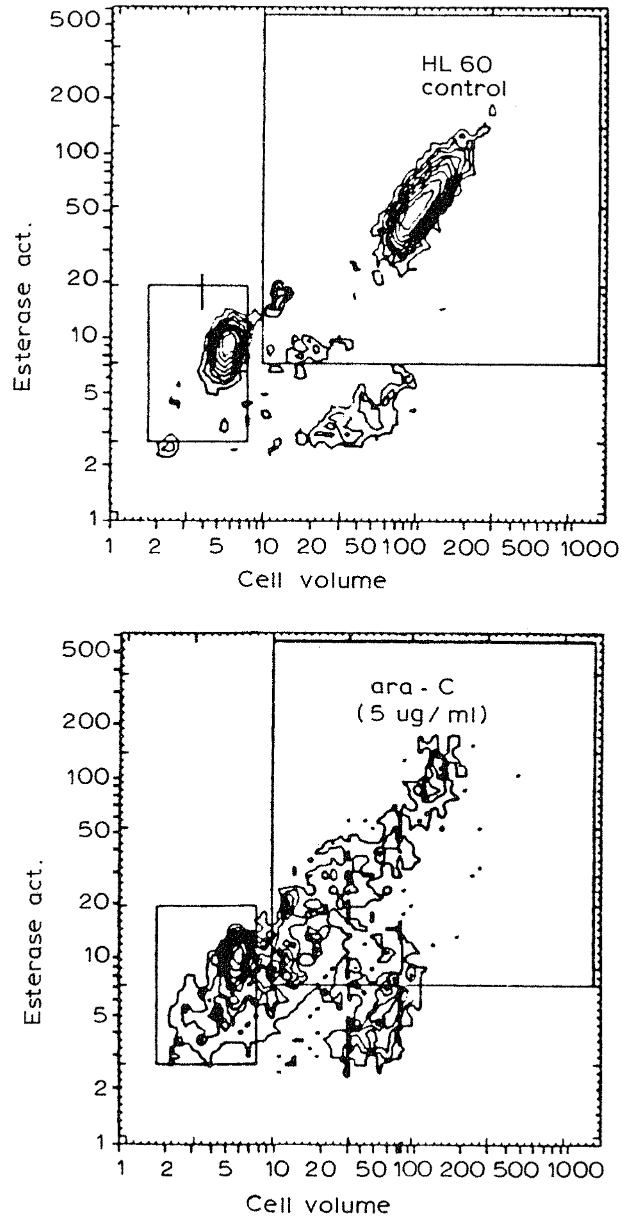

Fig. 2. Cell volume vs. esterase activity of HL60 cells incubated without cytostatic drug (above) and with ara-C (5 μg/ml) (below). The calibration particles are distinguishable from the cells by their smaller volume. 10 218 cells and particles were measured in the control incubation, as compared to 2 530 in the ara-C-treated culture. An estimation of the number of surviving cells compared to the control incubations without cytostatic drugs is possible from the ratio surviving cells/calibration particles. In this particular case, compared to the mean of four control assays, 3.5% of the cells survived.

tested ($P=0.19$). When the difference between T- and B-cell lines was tested under the influence of only ara-C, it was found to be significant, with a higher decrease in T-cell lines ($P=0.005$). HL60 also showed a significant decrease in pH_i (Table 2).

Cell volume

An increase of up to twice the original cell volume was seen under the influence of ara-C, daunorubicin and vincristine (Table 1). The highest volume increase of

Table 1 Esterase-concentration, cell volume and % surviving cells of the investigated cell lines as a function of the in-vitro cytostatic drug incubation. Shown are the parameter values of surviving cells in % of the mean of the four control incubations. The last column gives the range of the four control incubations. Values below 25% of the lowest control value or above 25% of the highest control value are printed with italic numbers. See Material and Methods for concentrations of cytostatic drugs. HL60, mean of two experiments on two different days. For all other cell lines the values of a single measurement is shown. The stability of fluorescence and cell volume was continuously monitored during the measurements.

	ara-C				L-Asparaginase				Daunorubicin				Prednisone				Vincristine				CO($n=4$) (range)
	10	1	0.1	0.01	10	1	0.1	0.01	10	1	0.1	0.01	10	1	0.1	0.01	10	1	0.1	0.01	
T-cells																					
MOLT4																					
Esterase	*11*	*14*	*46*	116	74	101	90	100	*25*	*16*	88	111	95	109	88	101	*60*	107	89	105	85–114
Volume	*168*	*163*	*155*	107	73	76	80	79	99	*137*	98	99	93	91	90	96	*146*	101	101	100	95–102
%surv	*21*	*35*	73	107	60	90	76	118	*20*	nt	*156*	125	*190*	*140*	111	90	93	93	120	143	78–122
JURKAT																					
Esterase	64	87	98	114	88	112	98	116	*49*	74	129	121	118	81	79	92	*61*	76	73	94	87–117
Volume	86	90	83	93	66	77	79	76	81	116	84	91	84	92	93	102	107	101	110	106	89–111
%surv	*22*	*39*	122	100	*28*	111	113	nt	5	*37*	103	nt	*31*	115	126	nt	*22*	*180*	100	nt	77–120
CEM																					
Esterase	*58*	81	64	110	73	98	86	104	*52*	127	92	110	87	108	87	97	87	105	81	87	85–112
Volume	*149*	*133*	*150*	103	78	74	74	77	107	85	85	89	81	84	84	100	97	98	99	111	93–106
%surv	*4*	*8*	*23*	96	*9*	120	106	95	*6*	nt	96	103	*52*	103	116	104	*34*	89	93	95	78–126
B-cells																					
RAJI																					
Esterase	94	86	104	110	82	94	104	112	70	98	89	103	99	101	90	102	87	102	96	86	93–107
Volume	*187*	*193*	*197*	121	72	101	98	98	122	*193*	*202*	117	79	96	93	101	*141*	134	109	110	85–112
%surv	*18*	*24*	*20*	*54*	*43*	77	79	92	*17*	*14*	*24*	71	92	81	92	95	*7*	*17*	*57*	92	82–110
RPMI1788																					
Esterase	106	96	106	101	97	109	105	99	64	93	103	101	106	112	96	116	90	104	107	106	98–101
Volume	*135*	*135*	115	106	89	100	102	102	117	121	133	105	96	93	91	97	123	116	99	105	97–103
%surv	*42*	*67*	*64*	85	*67*	*63*	78	102	*23*	*36*	*51*	*67*	86	64	77	69	*42*	*42*	93	83	96–105
DAUDI																					
Esterase	117	107	111	109	100	109	117	115	113	119	114	108	113	121	120	113	106	125	118	112	81–115
Volume	*141*	*134*	*137*	118	78	97	98	100	74	*142*	*135*	103	96	95	91	95	110	113	106	100	92–105
%surv	55	61	68	65	*53*	77	67	74	*38*	*52*	*56*	83	*60*	*57*	72	67	*44*	*43*	*57*	77	85–125
Myelocytic																					
HL60																					
Esterase	*133*	97	103	96	*136*	93	86	101	*132*	*150*	99	90	96	96	101	93	96	101	100	94	87–106
Volume	57	113	107	101	*32*	88	91	87	*38*	*35*	*186*	*151*	106	102	102	103	60	105	105	104	94–105
%surv	*3*	*35*	77	99	*1*	63	93	69	*0.2*	*0.6*	*23*	60	79	101	95	84	*2*	*37*	80	72	75–127

n.t., not tested.

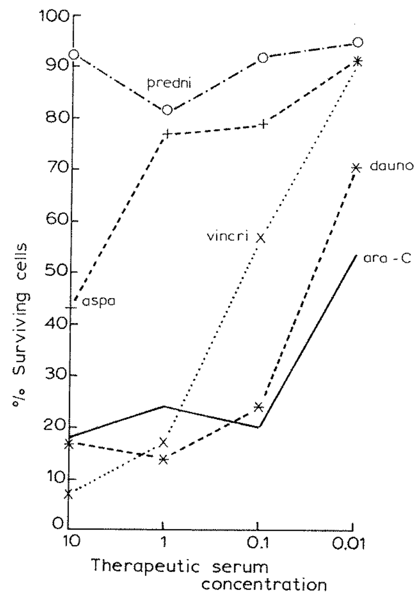

Fig. 3. Dose-response curves for the surviving cells from the B-cell line Raji in % of the mean of the four control assays without cytostatic drugs (= 100%). The range of the controls was 82–110%. See Materials and Methods for the concentrations of the cytostatic drugs.

Table 2 Changes of intracellular pH of surviving cells following in vitro incubation with cytostatic drugs. The values represent the differences between samples incubated at a 10-fold therapeutic serum concentration and the means of four control assays without drug. A negative value means acidifying of the sample. The values for the myelocytic line HL60 represent the measurements from two assays on different days. For all other cell lines the differences of a measurement of a single experiment are given. Values lower than 0.1 pH units of the lowest pH value of the control assays or higher than 0.1 pH units of the highest pH value of the control assays are printed with italic numbers.

Cell line	ara-C (5 μg/ml)	Aspa (2 U/ml)	Dauno (1 μg/ml)	Predni (100 μg/ml)	Vincri (0.1 μg/ml)	Controls (pH range)	Controls (mean pH)
T-cells							
Molt4	*−0.74*	−0.09	*−0.37*	0.01	*−0.26*	7.61–7.67	7.65
Jurkat	*−0.13*	0.02	−0.02	−0.02	−0.07	7.67–7.73	7.70
CEM	*−0.39*	*−0.21*	*−0.27*	−0.04	−0.04	7.82–7.88	7.84
B-cells							
RPMI1788	−0.08	−0.02	−0.05	0.00	−0.06	7.94–7.99	7.96
Daudi	−0.01	−0.02	−0.03	−0.08	−0.13	7.98–8.17	8.05
Raji	0.13	0.07	−0.03	0.14	−0.11	7.72–7.93	7.84
Myelocytic							
HL60 I	*−0.35*	*−0.57*	*−0.63*	−0.07	*−0.41*	7.79–7.94	7.88
II	*−0.15*	*−0.18*	*−0.60*	−0.03	*−0.32*	7.70–7.80	7.73

surviving cells was seen with myelocytic HL60 cells and the B-cell lines, especially Raji (Table 1). The influence on the cell volume was independent of the sensitivity of the cell line in terms of percentage surviving cells, and higher doses of cytostatic drugs usually produced more pronounced cell swelling (Table 1). Only daunorubicin was effective in inducing swelling in surviving HL60 cells. Swelling of these cells was best seen in cultures treated with concentration of 0.001 μg/ml (60% surviving cells) and 0.01 μg/ml (23% surviving cells), while 1 μg/ml caused no swelling of the 0.2% surviving cells. In the 0.01 μg/ml treated HL60, the mean cell volume was found to be 186% (167 and 212% in the second experiment, respectively) that of the controls (Table 1).

The T-cell lines showed less increase in cell volume than the B-cell lines ($P = 0.001$) (Table 1).

A decrease in cell volume was found in L-asparaginase-treated cultures, especially in T-cell lines. Furthermore, prednisone caused a slight decrease in cell volume in the T-cell lines CEM and Jurkat; higher doses of prednisone (100 μg/ml) caused a greater decrease (Table 1).

Discussion

Ideally, in vitro cytostatic drug assays should be performed before the individual patient is treated with cytostatic drugs. Therefore the assay should be fast and easy to carry out. The assay which currently best correlates with clinical data is the colony-forming assay [12]. A rapid flow-cytometric assay can be employed to screen for the number of surviving cells following cytostatic drug incubation [6]. A recent study in human leukemic cell lines showed a good correlation between a modified flow-cytometric assay and the stem cell assay, although the former measures the tumor cell kill, whereas the latter assesses the sensitivity of the tumor stem cell [13].

The present study was carried out so that the surviving cells (i.e., drug-resistant cells) can be further investigated by means of additional parameters incorporated into the assay; esterase concentration; cell volume; intracellular pH. The results show that the esterase concentration is significantly altered in the investigated T-cell lines, while smaller changes were found in B-cell lines ($P < 0.001$) (Table 1). One interpretation of these findings is that the decrease in esterase concentration is a precursor of cell death, while an alternative interpretation might be that due to the different cell origins (e.g., T- vs. B-cells), the two cell types react differently to treatment because of different mechanisms of counter regulation. The first interpretation is unlikely, since the cytostatic sensitive B-cell line Raji (Fig. 3) showed only minimal changes in terms of esterase concentration (Table 1).

A massive change in pH_i may cause cell death. The changes in pH_i observed in the present study were below one pH-unit. The maximum pH decrease (Molt4, ara-C 5 μg/ul) was 0.74 pH-units (Table 2). This probably represents a significant decrease in pH_i since the intracellular pH is regulated within narrow limits and the steady state of various enzymatic pathways may be drastically affected by shifts in intracellular pH.

T- and B-cells can be distinguished by means of their different behaviour following incubation in hypotonic solutions. Whereas T-cells that have swollen in hypotonic medium react with a fast decrease in cell volume when they are incubated in normotonic solution, B-cells remain swollen for much longer [14]. The results of this study show similar differences between T-and B-cell lines following incubation with cytostatic drugs. The B-cell lines investigated showed a significantly greater increase in volume under the influence of ara-C, daunorubicin and vincristine compared to the T-cell lines ($P = 0.001$) (Table 1). The promyelocytic line HL60 showed significant swelling only in the daunorubicin assay. Here, higher doses caused cell killing. An influence on HL60 cell volume could be found only in daunorubicin doses with more than 20% cell survival (Table 1). It is not clear whether this swelling is caused by altered ion gradients across the cell membrane or whether metabolic changes are the cause of cell swelling.

Flow cytometry has recently become a useful tool in in vitro cytostatic drug testing. The technique has been used to detect changes in DNA content [15, 16] and uptake of fluorescein-labelled cytostatic drugs such as a derivative of methotrexate [17] or the self fluorescent daunorubicin [18], and to determine the inhibtion of DNA synthesis following in vitro incubation with cytostatic drugs [19, 20]. Also flow cytometry has been used to determine cell killing after incubation with cytostatic drugs (6,7,13,21). Up to now, flow cytometry has not been used to determine changes in cell biochemical parameters of the surviving cells following in vitro incubation with cytostatic drugs.

The flow-cytometric cytostatic drug assay makes it possible to screen not only the number of surviving cells following in vitro cytostatic drug incubation but also their biochemical properties. This study was performed to establish the flow-cytometric methodology of in vitro cytostatic drug testing. Since it was performed on cell lines, we cannot exclude that the data are not representative for primary human leukemic cells and the testing of cell lines cannot replace the in vitro testing of the patient's cells. The results of the present study show, however, that the surviving cells of several human leukemic cell lines are significantly influenced by cytostatic drugs. Whether these are antemortem reactions or whether they are the expression of active defense mechanisms remains to be established. Flow-cytometric investigation of functional parameters may be of importance for determining the mechanisms of cytostatic drug resistance. These techniques can be readily performed on patient cells during the course of the disease.

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