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Toxicity of cytostatic drugs to normal bone marrow cells in vitro

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Abstract In this study we compared how different concentrations and periods of incubation of anthracyclines, amsacrine, and cytosine arabinoside would affect normal hematopoietic bone marrow cells in terms of interindividual differences in toxicity, the age of the donor, and the proliferative capacity of the bone marrow. Bone marrow was obtained from 36 donors in connection with bone marrow transplantation. After separation the mononuclear cell fraction was incubated with doxorubicin, 4-epidoxorubicin, daunorubicin, idarubicin, aclarubicin, mitroxantrone, amsacrine, and cytosine arabinoside for 1 h, for 3 h, or continuously. The cells were thereafter cultured in soft agar and CFU-GM were counted after 10–12 days. The results showed a large interindividual variation in toxicity for all drugs tested. Daunorubicin, idarubicin, aclarubicin, and mitoxantrone had a pronounced cytotoxic effect after 1 h of incubation. Doxorubicin and 4-epi-doxorubicin showed the greatest cytotoxic effect after 3 h and were also more toxic to normal bone marrow cells from donors over 40 years of age. Ara-C had a low cytotoxic effect after 1 and 3 h of incubation, even at high concentrations, but exerted a pronounced degree of toxicity during continuous incubation. Daunorubicin, idarubicin, and ara-C also showed increased toxicity to cell samples with a low proliferating capacity in the control. The conclusions drawn from these results are that interindividual variation, proliferation capacity, incubation conditions, and the age of the donors are factors of importance in the toxicity of drugs to normal hematopoietic bone marrow cells.

Key words Human CFU-GM · Anthracyclines · Ara-C · AMSA

Abbreviations *CFU-GM* Granulocyte macrophage colony-forming unit · *DOX* Doxorubicin · *EPI* 4-Epidoxorubicin · *DNR* Daunorubicin · *IDA* Idarubicin · *ACLA* Aclarubicin · *MIT* Mitoxantrone · *AMSA* Amsacrine · *Ara-C* Cytosine arabinoside · *FCS* Fetal calf serum · *CM* Conditioned medium

Introduction

In cancer treatment the toxicity of cytostatic drugs to normal hematopoietic bone marrow cells is generally the dose-limiting factor. This toxicity causes a significant number of deaths by infection or bleeding. In addition, fear of too much bone marrow toxicity may lead to suboptimal treatment of the tumor. Much of the development of new cytostatics focuses on reducing the bone marrow toxicity while maintaining the effect against the tumor cells, thus increasing the therapeutic index [23, 25]. It has also been suggested that different ways of giving these drugs may affect the toxicity [6, 13]. This has been shown for the antitumor effect of, e.g., cell-cycle-specific drugs [8]. However, little has been studied concerning the in vitro effects of cytostatic drugs on hematopoietic bone marrow cells, and what has been done has been performed mainly on samples from patients with various diseases [1, 11, 15, 28].

The antracycline antibiotic agents doxorubicin, 4-epidoxorubicin, daunorubicin, and idarubicin are intercalating agents that form complexes with topoisomerase II [24]. Aclarubicin is an anthracycline antibiotic that is suggested to inhibit RNA synthesis [22] but does not stimulate the formation of topoisomerase

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II-mediated cleavage [5]. The anthracenedione derivative mitoxantrone inhibits the formation of topoisomerase II activity [3]. The acridine derivative amsacrine intercalates with DNA and forms complexes with topoisomerase II and DNA [9]. Cytosine arabinoside (ara-C) is an antimetabolite that is active in the cell-cycle S phase after its phosphorylation to Ara-CTP [7].

Growth of CFU-GM is a well-established method for the study of normal hematopoietic progenitor cells in in vitro studies [4, 14, 15]. The main objective of this study was to evaluate possible interindividual differences with regard to the cytotoxicity of a cytostatic drug to normal bone marrow cells and to determine whether the age of the donor or the proliferating capacity of the bone marrow would be of importance for the differences in toxicity. The secondary aim was to compare the bone marrow toxicity of various anthracycline analogues and to study the effect of incubation concentrations and periods on the toxicity of different cytostatic drugs.

Materials and methods

Cells were obtained from 36 healthy donors (7–67 years old) for bone marrow transplantation. Bone marrow samples of 5–10 ml were collected in RPMI 1640 medium with preservative-free heparin; the mononuclear cells were immediately separated on sodium metrizoate/Ficoll (Lymphoprep; Nyegaard & Co, Oslo) [2] and were thereafter washed twice with RPMI 1640.

Cytostatic drugs

The cytostatic drugs used were the anthracyclines doxorubicin, 4-epidoxorubicin, and idarubicin, purchased from Pharmacia-Upjohn, and daunorubicin (Cerubidin), purchased from Rhône-Poulenc Rorer. Stock solutions of 100 μM were stored at −20 °C. Aclarubicin (Jaclacin) was purchased from Lundbeck AS; mitoxantrone (Novantrone), from Lederle/Cyanamid; and amsacrine (Amekrin), from Parke-Davis. Aclarubicin and amsacrine were stored in stock solutions of 100 μM at −20 °C, whereas mitoxantrone was freshly prepared for every incubation. Ara-C (Cytosar) was purchased from Pharmacia-Upjohn; stock solutions of 100 μM were made every week and stored at 4 °C.

Incubation procedure

The period of incubation with cytostatic drugs was 1 or 3 h in suspension culture or, as a continuous incubation, 10–12 days in soft agar. Mononuclear cells at a concentration of 5×10^5 cells/ml were incubated in RPMI 1640 with 10% FCS and 1% L-glutamine in polypropylene tubes in a shaking water bath at 37 °C. The final incubation volume was 1 ml, including the drugs that were added at 10-fold the final concentration. Concentrations used for short-term incubation were 0.2–1.0 μM for doxorubicin, 4-epidoxorubicin, and daunorubicin; 0.2 and 0.5 μM for aclarubicin; 0.05–0.2 μM for idarubicin and mitoxantrone; 0.5–1.0 μM for AMSA, and 1.0 and 5.0 μM for ara-C. For continuous incubation the cytostatic drugs were added directly to the agar culture at final concentrations as follows: doxorubicin, 4-epidoxorubicin, and aclarubicin, at 0.005 and 0.025 μM; daunorubicin and AMSA, at 0.0025–0.025 μM; idarubicin, at 0.0005–0.025 μM; and ara-C, at 0.025–0.05 μM.

The strategy was to incubate the cells with all drugs tested at at least two concentrations. Ten donors were tested against all drugs at all times but not at all different concentrations. When there was a shortage of material the experiments were scaled down. In those experiments we chose to incubate the cells during various periods for different drugs according to what we had previously shown to give an intracellular exposure mimicking the clinical situation after pharmacokinetics studies, e.g., 1 h for daunorubicin and continuous incubation for ara-C [20, 21]. For incubation periods and concentrations mimicking the in vivo situation we carried out a minimum of ten experiments. For incubation periods and concentrations leading to total cell kill, three experiments were regarded as sufficient.

Culturing methods

The soft-agar cultures were made with 1 ml in the feeder layer and 1 ml in the overlayer. The feeder layer consisted of one-third McCoy 2× with 30% FCS, one-third 1.5% agar, and 1/3 McCoy with CM (conditioned medium) [4], giving a final concentration of McCoy with 15% FCS and 0.5% agar. We used the same batch of CM in all experiments, which for these purposes renders the stimulatory properties similar to those of standardized GM-CSF. The final concentration in the overlayer was McCoy with 15% FCS, 1×10^5 cells/ml, and 0.3% agar. When the incubations had been completed the samples were centrifuged (400 *g* for 10 min) and the supernatants were discarded. Without being washed, the cells were resuspended in 5 ml agar-medium, 1 ml was seeded out as the overlayer in each of the three 35-mm culture dishes (Falcon 3001).

Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for 10–12 days. CFU-GM comprising more than 40 cells were counted and the cytotoxic effect was expressed as the percentage of a drug-free control value. A significant effect was defined as ≤60% colonies relative to the amount of colonies in the control, which was regarded as 100%.

Statistical analysis

Wilcoxon's signed-rank test and Mann-Whitney's rank-sum test were used to evaluate differences between paired values of sensitivity. A P value of < 0.05 was regarded as statistically significant.

Results

Untreated controls

The mean number of colonies counted in the controls cultured without cytostatic drugs were 127 ± 71 (range 21–312) colonies/dish. There was no significant correlation between the age of the donor and the proliferating capacity of the bone marrow ($r^2 = 0.097$). Controls from donors under 40 years of age formed 134 ± 74 colonies as compared with 105 ± 65 colonies for donors over 40 years of age (not significant, NS). There was also no significant difference between sex and colony growth. Mean numbers of colonies were 121 ± 63 for women and 124 ± 78 for men (NS).

Incubation for 1 h

The mean values recorded for CFU-GM after 1 h of incubation with different concentrations of anthracyclines and cultivation in soft agar are shown in Table 1. This incubation revealed marked differences between the anthraquinones. For doxorubicin and 4-epidoxorubicin an incubation concentration of 0.5 μM was necessary to

Table 1 Mean values recorded for CFU-GM after 1 h of incubation in different concentrations of anthracyclines and mitoxantrone followed by cultivation in soft agar. All data are expressed as percentages of untreated control values

μ*M*	DOX	*n*	EPI	*n*	DNR	*n*	ACLA	*n*	IDA	*n*	MIT	*n*
0.05	–		–		–		–		27 ± 29	12	16 ± 20	19
0.2	62 ± 37	5	82 ± 12	5	26 ± 24	24	40 ± 31	12	1 ± 1	3	0	4
0.5	30 ± 20	6	46 ± 19	5	1 ± 1	13	0	12				

Table 2 Mean values recorded for CFU-GM after 3 h of incubation in different concentrations of anthracyclines followed by cultivation in soft agar. All data are expressed as percentages of untreated control values

μ*M*	DOX	*n*	EPI	*n*	DNR	*n*	ACLA	*n*	IDA	*n*
0.05	–		–		–		–		26 ± 36	5
0.2	36 ± 29	12	53 ± 37	12	6 ± 6	5	42 ± 35	5	0	3
0.5	9 ± 11	12	17 ± 17	12	0	4	0	4	–	

Table 3 Mean values recorded for CFU-GM after continuous incubation in different concentrations of anthracyclines during cultivation in soft agar. All data are expressed as percentages of untreated control values

μ*M*	DOX	*n*	EPI	*n*	DNR	*n*	ACLA	*n*	IDA	*n*
0.005	76 ± 13	8	75 ± 27	7	58 ± 27	10	85 ± 15	7	52± 37	7
0.025	24 ± 18	10	25 ± 15	8	0 ± 0	4	34 ± 33	8	0 ± 0	3

achieve significant toxicity. At this concentration, almost all cells were killed by daunorubicin, aclarubicin, idarubicin, and mitoxantrone. As compared with the effect of doxorubicin and 4-epidoxorubicin at 0.5 μ*M*, a similar effect was achieved by daunorubicin and aclarubicin at a concentration of 0.2 μ*M*, although daunorubicin was significantly more toxic than aclarubicin ($P < 0.05$). For idarubicin and mitoxantrone, 0.2 μ*M* was sufficient to kill all cells, and at an incubation concentration of 0.05 μ*M* a high level of toxicity was demonstrated.

Incubation for 3 h

With this incubation period the differences were smoothed out and a cytotoxic effect of doxorubicin and 4-epidoxorubicin was seen at a concentration of 0.2 μ*M*, although 4-epidoxorubicin was significantly less toxic than doxorubicin ($P < 0.05$; Table 2). As found for the 1-h incubation period, the toxicity of daunorubicin, aclarubicin, and idarubicin was higher than that of 4-epidoxorubicin, although these differences were not statistically significant.

Continuous incubation

The difference seen between the toxicity of daunorubicin and idarubicin in the short-term incubations disappeared after continuous incubation (Table 3). As compared with daunorubicin, doxorubicin and 4-epidoxorubicin were less toxic ($P < 0.05$, respectively). 4-Epidoxorubicin was also less toxic than idarubicin ($P < 0.05$). In contrast to the short-term incubations, the toxicity of aclarubicin at both concentrations was similar to that of doxorubicin and 4-epidoxorubicin.

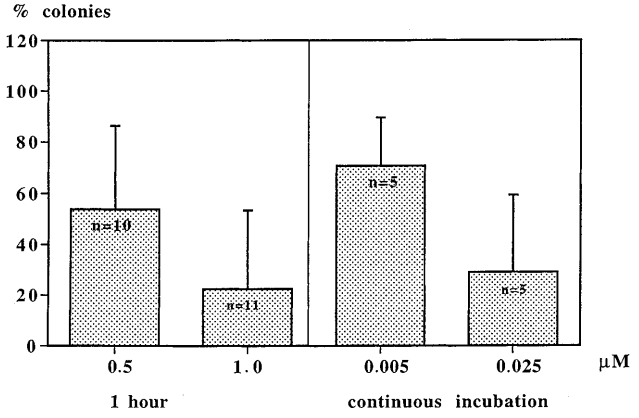

Fig. 1 Toxicity of AMSA to normal bone marrow cells in vitro during different periods of incubation at different concentrations

AMSA cytotoxicity

The cytotoxicity of AMSA incubated for 1 h and as a continuous incubation is shown in Fig. 1. Continuous incubation of AMSA at 40- to 100-fold lower concentrations was comparable with the short-term incubation with regard to the toxic effects.

Ara-C cytotoxicity

Figure 2 shows the cytotoxicity of ara-C in short-term and continuous incubations. The cytotoxic effect in the short-term cultures was low despite the incubation

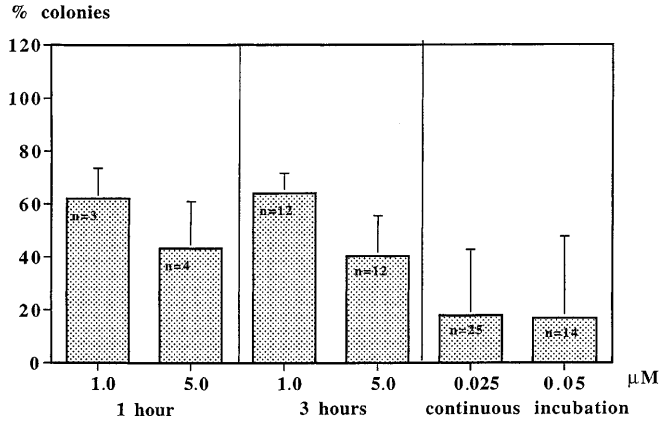

Fig. 2 Toxicity of ara-C to normal bone marrow cells in vitro during different periods of incubation at different concentrations

concentrations. In contrast, 40- to 100-fold lower concentrations exerted a pronounced effect during continuous incubation.

Concentrations resembling the in vivo situation

Figure 3 shows the difference in cytotoxicity between incubations that give intracellular concentrations mimicking the clinical situation [20, 21]. At these incubation times and concentrations a pronounced interindividual variation in toxicity was found for these drugs. Daunorubicin at a concentration of 0.2 µ*M* incubated for 1 h was significantly more toxic than both 4-epidoxorubicin incubated for 3 h and aclarubicin incubated for 1 h ($P < 0.05$). Mitoxantrone was significantly more toxic than daunorubicin and aclarubicin in spite of its 4-fold lower incubation concentration ($P < 0.05$).

Cytostatic drugs in relation to age

Bone marrow cells from donors over 40 years of age was more sensitive to some anthracyclines as opposed to bone marrow cells from donors under 40 years of age. Cells from donors under 40 years that were incubated with 0.2 µ*M* doxorubicin for 3 h formed 57 ± 25% colonies relative to the untreated control, whereas those from donors over 40 years of age formed 5 ± 6% colonies relative to the untreated control ($P < 0.05$). The corresponding values recorded for 0.5 µ*M* doxorubicin were 15 ± 15% versus 2 ± 3% colonies ($P < 0.05$). The cytotoxic effect of 4-epidoxorubicin at 0.2 and 0.5 µ*M* was 83 ± 20% and 30 ± 1% colonies, respectively, for donors under 40 years and 9 ± 6% and 1 ± 2% colonies, respectively, for donors over 40 years ($P < 0.05$). Moreover, 0.05 µ*M* ara-C in continuous incubation was marginally more toxic to bone marrow cells from donors over 40 years, which formed 9 ± 12% colonies versus 25 ± 30% colonies for donors under 40 years of age ($P = 0.066$).

Cytostatic drugs in relation to cell growth

Continuous incubation with 0.05 µ*M* ara-C showed significantly higher toxicity to bone marrow cells that formed <100 colonies in the control. The mean number of colonies was 31 ± 39% for samples with >100 colonies in the control as compared with 3 ± 4% for samples with fewer than 100 colonies ($P < 0.05$). Also, daunorubicin and idarubicin appeared to be more toxic to bone marrow cells that formed <100 colonies in the controls. The mean toxic effect of 0.2 µ*M* daunorubicin incubated for 1 h was 18 ± 16% colonies from a control

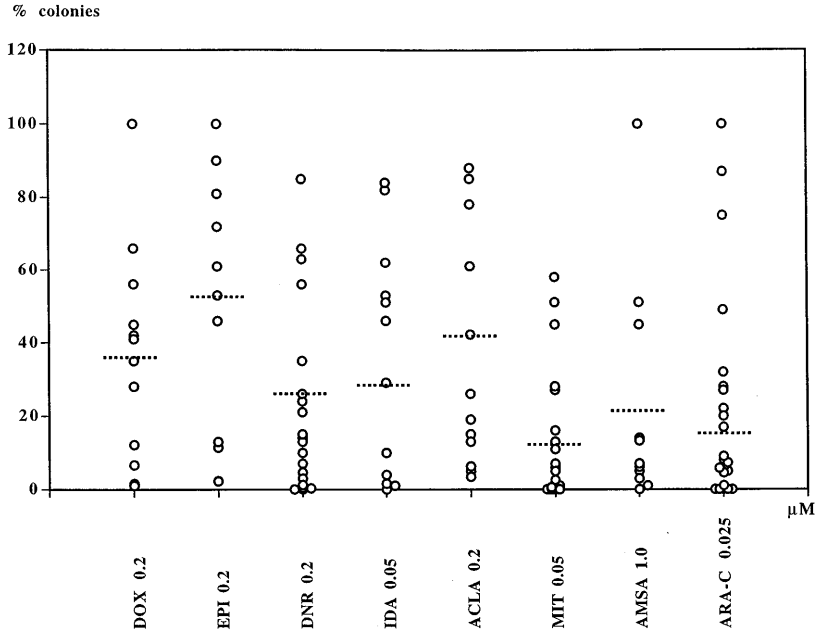

Fig. 3 Toxicity of different cytostatic drugs to normal bone marrow cells in vitro during incubation with concentrations mimicking the in vivo situation. Incubations with DOX and EPI were performed at a concentration of 0.2 µ*M* for 3 h. Incubations with DNR and ACLA were performed at concentrations of 0.2 µ*M* for 1 h. Incubations with IDA and MIT were performed at a concentration of 0.05 µ*M* and those with AMSA at 1 µ*M* for 1 h. Ara-C was incubated at 0.025 µ*M* continuously. Mean values are indicated by *dotted lines*

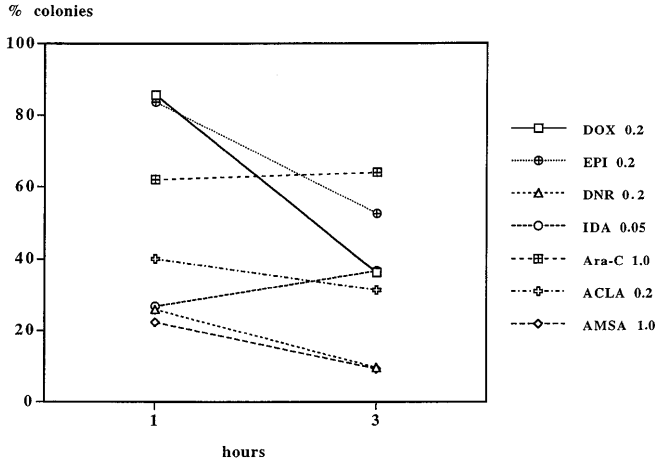

Fig. 4 Mean toxicity of different cytostatic drugs incubated for 1 and 3 h

that formed <100 colonies versus 36 ± 30% colonies from a control forming >100 colonies (NS). For 0.05 μ*M* idarubicin the corresponding values were 8 ± 9% colonies as compared with 41 ± 31% colonies (NS).

Impact of incubation time

Drugs with a rapid intracellular uptake, such as daunorubicin, idarubicin, AMSA, or aclarubicin, exerted a highly cytotoxic effect on the bone marrow after 1 h, which did not change markedly after 3 h as shown in Fig. 4. Doxorubicin and 4-epidoxorubicin showed increased toxicity (significant only for doxorubicin; $P < 0.05$) after 3 h as compared with 1 h of incubation. Ara-C, which is usually given as a continuous or repeated infusion, had a limited effect in short-term incubations, although its incubation concentration was 40-fold that used in continuous incubations.

Discussion

The aim of the investigation was to evaluate interindividual differences and to compare the toxicity of various cytostatics to normal hematopoietic bone marrow cells. The results showed pronounced interindividual differences in the toxicity of a single drug when tested against bone marrow cells from different donors. The cytotoxic effects observed at these incubation concentrations varied by a factor of 50. These differences cannot be explained by variations in the technical procedure, as it was uniform throughout and triplicates were made of all samples. In addition, since we used the same CM in all experiments, it is unlikely that the interindividual variations were due to differences in stimulatory capacity. Thus, the interindividual variations appear to depend on real differences in the sensitivity of normal progenitor cells to cytostatic drugs. This is suggested by the findings of similar relative intraindividual sensitivity when bone marrow from one donor was incubated with different anthracycline analogues.

Calculations of IC_{50} values, as is normally done with cell lines, would have been ideal for purposes of comparison. However, that would have at least doubled the number of experiments, which was not possible due to limitations of material. Our incubation concentrations differ from those of some other investigators. The incubation concentrations necessary to kill a certain amount of the cells may vary between different laboratories, depending on the methods used or the source of the cells. In our laboratory we have experienced that methods measuring only cell kill, such as the bioluminescence ATP assay or the MTT assay, give a lower degree of sensitivity as compared with methods by which cell growth is measured, such as the colony assay. It is also difficult to compare the toxicity of cytostatic drugs to leukemic cells as determined by one method with their toxicity to normal cells as measured by another method.

On the basis of their clinical effects, anthracyclines can be divided into two groups, one affecting only hematologic malignancies and the other affecting solid tumors as well. The former group includes drugs such as daunorubicin, idarubicin, and aclarubicin and the latter, drugs such as doxorubicin and 4-epidoxorubicin. In previous studies we showed that these differences could be related to the physical properties of a particular drug, such as lipid solubility and affinity to DNA, that affect the intracellular peak concentration and retention [12]. Doxorubicin and 4-epidoxorubicin have low lipid solubility and take 3 h or more to reach intracellular steady-state levels. The pronounced effect of daunorubicin, mitoxantrone, aclarubicin, and idarubicin after 1 h of incubation is in accordance with the rapid intrusion and high intracellular peak concentrations of these drugs. The intracellular accumulation of idarubicin is 4 times that of daunorubicin incubated at the same concentration [27]. In short-term incubations, drugs with a slow rate of intracellular accumulation, such as doxorubicin and 4-epidoxorubicin, were less toxic than, e.g., daunorubicin, but this difference was minimized by continuous incubation. The drug concentrations used in the continuous incubations were about 40 times lower than those used in the short-term incubations, yet there were marked interindividual differences in toxicity. This finding is in accordance with the report of Raijmakers et al. [15], who showed that the cytotoxic effect to CFU-GM was directly correlated with tightly bound doxorubicin, regardless of the incubation period.

The toxicity of AMSA in short-term incubations was comparable with that in continuous incubations at 40- to 100-fold lower incubation concentrations. For Ara-C, the toxicity of the short-term incubations was low even at high concentrations, but in continuous incubations the toxicity was pronounced at a 200-fold lower concentration. This importance of continuous incubation for the effects of ara-C is in accordance with previous uptake studies [10, 14], and that a continuous or repeated infusion is necessary to reach toxicity is well

known in treatment with cells-cycle-specific drugs such as ara-C [8]. Spiro et al. [18, 19] found similar toxicity for AMSA in normal bone marrow CFU-GM and in colony-forming cells from chronic myelocytic leukemia (CML) patients, whereas the toxicity of ara-C revealed increased sensitivity of the CML colony-forming cells relative to the normal CFU-GM.

The continuous incubation could be regarded as a long-term incubation. Different drugs may have different degrees of stability, which influences the actual level of exposure to the drug during long-term cultivation; thus, our results cannot be transformed to a continuous infusion of the drug in vivo [17].

In the last part of this study we focused especially on incubation periods and concentrations that we had previously shown to give toxic effects and intracellular concentrations mimicking the in vivo pharmacokinetics in leukemic cells after infusion of anthracyclines and ara-C [20, 21]. These concentrations and times have been shown to have a good predictive value on leukemic cells as determined with the DiSC assay [16, 26]. In the present study these incubations also showed pronounced interindividual differences, with a clear distinction being observed between high and low levels of in vitro toxicity to normal bone marrow cells. Our results indicate that these differences might be influenced by age and proliferating capacity and may support the clinical observation that elderly patients have difficulties in tolerating treatment with cytostatic drugs.

It is difficult to compare the in vitro toxicity of cytostatic drugs to normal bone marrow cells with the effect on tumor cells in the same patient using the same method. Some attempts have been made to compare the effect on leukemic cells with the toxicity to normal cells from individuals without hematologic malignancies as evaluated by different methods. Park et al. [11] showed a higher probability of complete responses in patients whose leukemic cells were more sensitive to cytostatic drugs than were normal bone marrow cells tested simultaneously. Ajani et al. [1] found doxorubicin to be more toxic to normal bone marrow CFU-GM than to tumor cells grown in liquid culture.

Cytostatic treatment is hampered by the toxicity of the agents to normal hematopoietic bone marrow cells. The pronounced interindividual differences in sensitivity to cytostatic drugs make attractive the concept of developing a method for testing patients' normal bone marrow cells against the drugs intended for use.

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