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In Vivo Cellular Adriamycin Concentrations Related to Growth Inhibition of Normal and Leukemic Human Bone Marrow Cells

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Abstract—*Inhibition of clonogenicity of normal and leukemic human hematopoietic progenitor cells was studied after* in vivo *and* in vitro *exposure of bone marrow to adriamycin (ADM). Flow cytometric determination of cellular ADM concentrations in blast cells, expressed in fluorescence units/cell (FU/cell), correlated well with the extent of cytotoxicity. After* 2 h in vitro *exposure to* 500 ng *ADM/*ml, *the ADM concentration of leukemic* (n = 7) *and normal* (n = 4) *bone marrow blast cells amounted to* 231 ± 180 *and* 249 ± 53 *FU/cell respectively, producing moderate decreases in clonogenicity by* 44 ± 30 *and* 54 ± 27%. *Exposure to* 2000 ng/ml *produced ADM concentrations of* 1184 ± 472 *FU/cell for leukemic blast cells and* 1024 ± 281 *FU/cell for normal blast cells. Inhibition of clonogenicity was* 96 ± 7% *in leukemic blasts and* 99 ± 1% *in normal blasts.*

In vivo *ADM concentrations in leukemic blast cells at* 1–2 h *after administration were* 216 ± 98 *FU/cell* (n = 8 *patients). This implies that inhibition of clonogenicity after administration of conventional dosages of ADM will be approx.* 60–70% *for both leukemic and normal bone marrow progenitor cells. Such values were noted in four patients of whom bone marrow was cultured, which was obtained shortly after ADM monotherapy.*

INTRODUCTION

ANTHRACYCLINES have been incorporated in most standard regimens for remission induction therapy in adult acute nonlymphocytic leukemia [1]. Plasma pharmacokinetic studies have revealed a large drug distribution volume consistent with considerable drug accumulation in extravascular compartments [2, 3]. Cellular anthracycline concentrations were 200- to 800-fold higher than those observed in plasma [4–6]. Attempts to correlate plasma anthracycline concentrations to cellular drug levels [5, 6], and to correlate plasma levels with the ultimate clinical result [4, 7, 8] have been only partially successful.

Cellular adriamycin (ADM) concentrations occurring *in vivo* after drug administration showed retention of the drug for several days [5, 6]. Similar intracellular drug persistence was observed after *in vitro* incubation [9].

In this study normal and leukemic blast cells from human bone marrow were exposed *in vitro* to ADM concentrations. Toxicity to colony growth capacity was determined. The concentration range chosen produced cellular drug concentrations of the same order as those occurring *in vivo* after ADM administration. Thus, an attempt was made to relate inhibition of leukemic and normal clonogenic cells to cellular ADM concentrations achieved *in vivo* with conventional drug dosage schedules.

PATIENTS, MATERIALS AND METHODS

Chemicals

ADM was obtained from Roger Bellon (Neuilly sur Seine, France). Pure ADM and ADMol for chromatographic purposes were kindly provided by Prof. Dr. F. Arcamone (Farmitalia Carlo Erba, Milan, Italy). All further chemicals used were obtained from Merck (Darmstadt, F.R.G.) and were of analytical grade.

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Bone marrow and blood sampling, and storage

For *in vitro* studies, bone marrow was aspirated from seven newly diagnosed patients with acute nonlymphocytic leukemia and from four patients with normal bone marrow (undergoing thoracic surgery). For determination of the range of cellular ADM concentrations achieved *in vivo*, blood and bone marrow cells of patients with acute nonlymphocytic leukemia were obtained during remission induction treatment ($n = 8$) or maintenance therapy ($n = 3$) with ADM. ADM was administered on 3 consecutive days at 30 mg/m^2/day (and at day 1 only during maintenance), together with cytosine arabinoside 200 mg/m^2/day for 7 days.

For determination of inhibition of clonogenicity after *in vivo* ADM monotherapy bone marrow was obtained for four patients: two with leukemia in remission, one with Hodgkin's disease without bone marrow infiltration and one with chronic myeloid leukemia in blast crisis. Additional chemotherapy was resumed after the last sample was taken. All patients gave informed consent for participation in these studies. Bone marrow was collected in acid citrate dextrose (ACD-A buffer, pH 7.4) on ice and filtered through a 70 μm filter. Peripheral blood admixture in the suspension was determined [10] to exclude samples with more than 20% admixture of nucleated blood cells. For use in clonogenic assays cells were washed with Hanks' Balanced Salt Solution (HBSS) and layered on a Percoll cushion (density 1.085 g/ml, pH 7.4, 300 mOsm/kg). Cells from the interface were washed twice, and resuspended in Dulbecco's minimal essential medium (DMEM) supplemented with 20% fetal calf serum. The cell suspension was diluted 1:1 with 20% (v/v) dimethylsulfoxide in DMEM. They were cryopreserved using a temperature controlled freezing procedure [11], and stored in liquid nitrogen.

Since leukemic and normal cells were obtained at different times, cryopreserved bone marrow was used in order to enable assessment of inhibition of clonogenicity simultaneously after exposure to ADM. This eliminates the influence of day-to-day variation in culturing conditions. In pilot studies the clonogenic efficiency of fresh and cryopreserved bone marrow were comparable.

In vitro *ADM exposure*

Cryopreserved bone marrow cells were rapidly thawed, incubated with DNase for 10 min, washed, counted and resuspended in Tris-buffered DMEM supplemented with 10% fetal calf serum at a final cell concentration of 10^5 cells/ml [11]. After 30 min preincubation, ADM was added to final concentrations ranging from 50 to 2000 ng/ml. At the end of the 2-h incubation a sample was taken for flow cytometric determination of the ADM concentration in blast cells as described below. Cells were washed twice with cold HBSS and resuspended in culture medium. Cellular ADM concentration was determined again just prior to plating. This procedure revealed how much cellular ADM was lost during the washing procedure, and it provided the actual cellular (tightly bound) drug concentrations at the moment of plating.

Clonogenic assays

The assay of the committed myeloid progenitor cells for normal (CFU-GM) and leukemic (CFU-L) bone marrow cells has been described previously [12]. All assays were performed in duplicate in 35 mm Petri dishes containing 2 ml of the cell suspension. Optimal cell concentrations had been determined previously in order to obtain approx. 100 colonies in the control dish. The cells were resuspended in DMEM. Supplemented wih 20% (v/v) fetal calf serum, 5% (v/v) placenta conditioned medium as a source of colony stimulating factor and 0.3% (w/v) Bacto agar (Difco, Detroit, Michigan, U.S.A.).

Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. After 12 days cell-aggregates consisting of 40 cells or more were scored as colonies.

Collection of patient samples

The procedure of sample collection has been described previously [13]: bone marrow samples were collected in cold ACD-A and processed as described above. The remaining erythrocytes and normoblasts were lyzed with ammonium chloride. The white cells were resuspended in cold NaCl solution (0.9%). One aliquot of the suspension was stored at −20°C for analysis with high performance liquid chromatography (HPLC), another aliquot was kept on ice for flow cytometric determination of cellular ADM concentration. Blood samples were drawn in heparinized polypropylene tubes on ice. Plasma was removed and stored at −20°C until analysis. Erythrocytes in the pellet were lyzed and the white cells were processed as described above.

Laser flow cytometric (FCM) determination of blast cellular ADM concentration

Cellular ADM concentrations in blast cells were determined with three-parameter laser flow cytometry as has been described previously [13]. Briefly: cell samples were measured using a 50H Cytofluorograph (Ortho Diagnostic Systems Inc, Westwood, MA, U.S.A.), equipped with a 5 W argon laser exciting at 488 nm with an output of 500 mW. The fluorescence voltage setting was at approx. 590 mV. Calibration was performed each day using fluorescent microspheres (Fluoresbrite, Polysciences, Warrington, PA, U.S.A.). Data were stored in list mode and analyzed on a PDP 11-34 (Digital

Electronics Company, Maynard, MA, U.S.A.). Combination of forward and perpendicular light scatter signals enabled identification of cell subsets such as blast cells, lymphocytes, myeloid cells and cell debris [13]. Cells from each subpopulation were sorted to verify their identity microscopically. Fluorescence was recorded on a linear scale from 0 to 8192 channels [13], without alterations in gain, to detect low vs. high cellular ADM concentrations. Cellular ADM concentrations were quantitatively determined in cells of distinct subpopulations like the blast cell subset [13]. They were expressed in arbitrary fluorescence units per cell (FU/cell), representing the mean fluorescence per cell from a selected subpopulation (referring to the channel number). Fluorescence data given were corrected for autofluorescence by subtraction of the blank value. Of each sample at least 10,000 cells were analyzed at a flow-rate of 1000 cells/s. Cellular ADM concentrations obtained by flow cytometry correlated well with the results of the determination by HPLC ($r = 0.99$, $n = 9$) [13]; 100 FU/cell appeared to correspond with approx. 4 fg/cell.

HPLC analysis of cellular and plasma ADM content

The method was described previously [6]. Briefly: to 250 µl of the cell suspension or to 500 µl plasma, 3 ml chloroform/methanol 9:1 (v/v) and 100 µl Tris buffer (1 M, pH 8.7) was added. After two extractions a 500 µl sample was injected on a Lichrosorb 5Si60 silica column (100 × 3 mm inner diameter). Detection was carried out using a fluorometer with excitation wavelength of 488 nm and emission above 550 nm. The detection limit of ADM and adriamycinol was 1 ng.

Statistical analysis

Data obtained from the clonogenic assays and from measurements of cellular ADM concentrations were analyzed for statistical significant differences using the Wilcoxon 2-sample test. Only significant differences have been indicated in the text.

RESULTS

Flow cytometric discrimination of the blast cell population

Multiparameter flow cytometry of normal and leukemic bone marrow samples enabled discrimination of the blast cell subpopulation based on their cellular forward and perpendicular light scatter signals. The higher the percentage of blast cells in the suspension, the higher the percentage of blast cells in the scatter-defined rectangle indicated in Fig. 1. In a normal bone marrow sample containing 1–2% blast cells, over 60% of the blast cells were found in the cell scatter rectangle indicated in Fig. 1A, together with some erythroblasts, monocytes and non-blastic myeloid cells. In leukemic bone marrow with more than 20% blast cells, nearly 100% of the sorted cells in the scatter window were blast cells (Fig. 1C). Moreover, flow cytometry allows the study of viable cells separately from dead cells and cell debris. Especially when cryopreserved cells are used, the amount of dead cells and debris can be relatively high (Fig. 1B and C). This can lead to overestimation of ADM concentrations in viable blast cell populations, since these dead cells and the debris contained considerably more ADM (data not shown).

Cellular ADM concentration in vitro

Cellular ADM concentrations in blast cells were determined both at the end of the *in vitro* ADM exposure and just prior to plating. For both leukemic and normal bone marrow cells, a linear correlation existed between extracellular ADM concentrations and blast cell ADM concentrations in the concentration range tested ($n = 8$, all $r > 0.95$), which implies an essentially non-saturable cellular ADM uptake in this range. After washing just prior to plating a proportional loss of 48 ± 11% of the initial cellular ADM was observed over the ADM concentration range tested. This indicates a free cellular ADM fraction of approx. 48% which was removed during the washing procedure. The remaining tightly bound cellular ADM concentrations at the moment of plating were plotted against the growth inhibition (Fig. 2A).

Clonogenicity and cellular ADM concentration in vitro

After exposure of marrow to 100 ng ADM/ml, clonogenicity of leukemic cells was depressed by 20 ± 17% at cellular ADM concentrations of 76 ± 41 FU/cell (Table 1). Moderate inhibitory levels of 44 ± 30% were observed after 2 h *in vitro* exposure to 500 ng ADM/ml (Fig. 2B), which produced cellular drug concentrations of 231 ± 180 FU/cell (Fig. 2A). At extracellular concentrations of 2000 ng/ml, both normal CFU-GM and leukemic CFU-L (Fig. 2B) were almost completely inhibited (leukemic cells: 96 ± 7% and normal cells: 99 ± 1%) at ADM concentrations in the blast cells of 1184 ± 472 FU/cell (normal bone marrow 1023 ± 281 FU/cell, Fig. 2A). The calculated IC_{50} (the drug concentration at which 50% inhibition of clonogenicity was expected) for leukemic cells was reached at 230 ± 102 FU/cell after 2 h exposure to 545 ± 252 ng/ml. The calculated IC_{90} values were 936 ± 240 FU/cell after exposure to 1146 ± 166 ng/ml for leukemic bone marrow, and 679 ± 155 FU/cell after exposure to

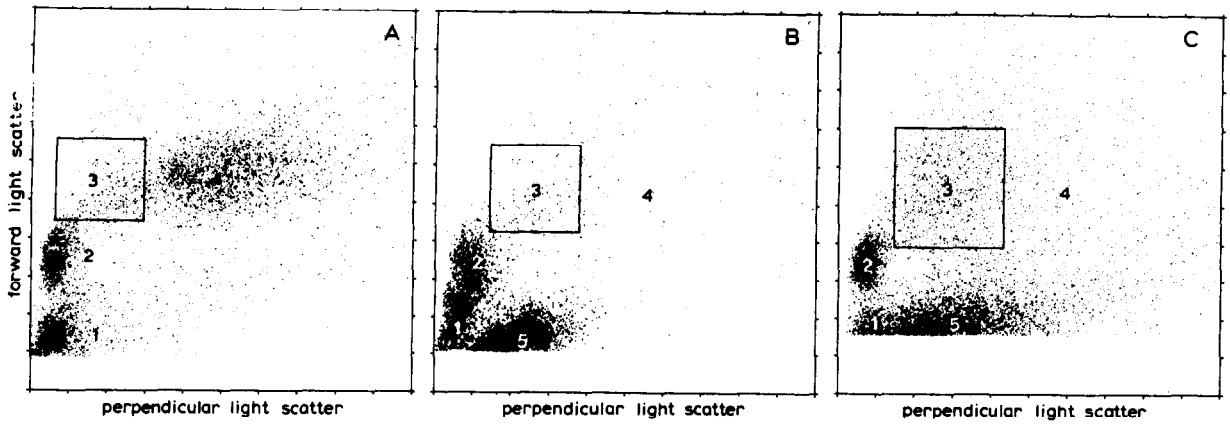

Fig. 1. Two-parameter (forward and perpendicular) light scatter plot of normal fresh bone marrow (A), cryopreserved normal bone marrow (B) and cryopreserved bone marrow from a patient with acute nonlymphocytic leukemia (C). The blast cell subpopulations are indicated by rectangle number 3. Note the amount of dead cells (population 1) and cell debris (population 5) in the cryopreserved bone marrow samples. Population 2 consists of lymphocytes and population 4 of mature myeloid cells.

Table 1. Measured blast cell adriamycin concentrations after in vitro *exposure, related to inhibition of clonogenicity*

Media adriamycin concentration (ng/ml)	Blast cell adriamycin concentration (FU/cell, mean ± S.D.) Leukemic $n = 7$	Normal $n = 4$	Inhibition of clonogenicity (control set at 0%) (%, mean ± S.D.) Leukemic $n = 7$	Normal $n = 4$
50	48 ± 68	35 ± 34	12 ± 16	11 ± 10
100	76 ± 41	76 ± 46	20 ± 17	8 ± 9
500	231 ± 180	249 ± 53	44 ± 30	55 ± 27
1000	850 ± 455	641 ± 112	86 ± 13	94 ± 6
2000	1184 ± 472	1023 ± 281	96 ± 7	99 ± 1

Normal and leukemic human bone marrow cells were incubated with indicated concentrations of adriamycin for 2 h. Blast cell adriamycin concentrations (FU/cell) were determined just before plating by means of flow cytometry. 12 days after plating colonies were scored. Control cultures had 102 ± 12 colonies per 10^5 plated cells for normal cells and 94 ± 15 (range 71–112) colonies per plate for leukemic cells.
No significant differences were noted in cellular adriamcyin concentrations or inhibition of clonogenicity between leukemic and normal cells at the different media concentrations.

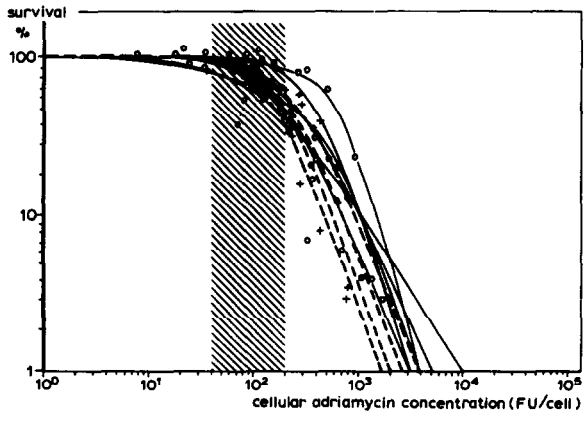

Fig. 2. A. Inhibition of clonogenicity plotted vs. bound cellular adriamycin concentrations. The hatched area represents the tightly bound cellular adriamycin concentrations observed in blast cells 1–2 h *after* in vivo *adriamycin administration during* 1, 2 *and* 3 *consecutive days resulting in FU/cell of* 62 ± 20, 97 ± 43 *and* 122 ± 50 *respectively.*

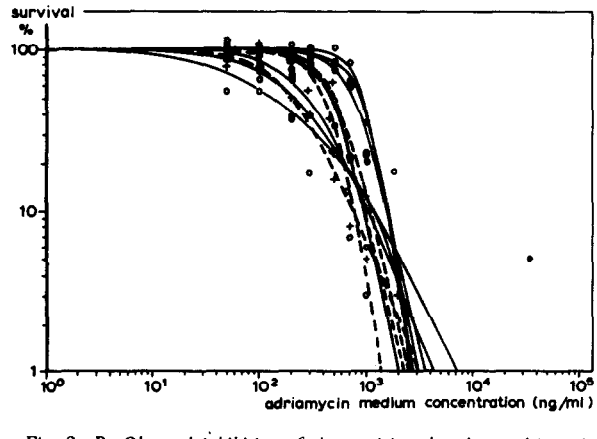

Fig. 2. B. Observed inhibition of clonogenicity plotted vs. adriamycin concentration in the medium. Incubation for 2 h. +: *Normal bone marrow (fresh and thawed)* +, *Broken lines.* ○: *Leukemic bone marrow.* ●: *Patient with resistant leukemia, solid lines.*

Table 2. In vivo *observed cellular adriamycin concentrations*

	Peak concentration (FU/cell)		Plateau concentrations (FU/cell)	
	Leukemia	Normal	Leukemia	Normal
(*n*)	8	3	8	3
Day 1	96 ± 29	140 ± 69	62 ± 20	69 ± 21
Day 2	173 ± 58		97 ± 43	
Day 3	216 ± 98		122 ± 50	

Concentrations were measured at 5 min after the injection (peak concentration) and 1–2 h thereafter (plateau concentration). Adriamycin was administered as 30 mg/m²/day on consecutive days to leukemia patients (n = 8) and on day 1 to patients in remission (n = 3).

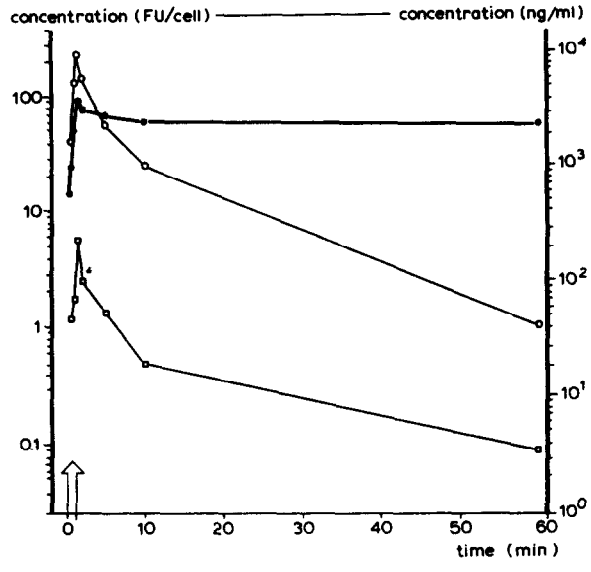

Fig. 3. Cellular and plasma adriamycin concentration–time curve during the first 60 min *after* 55 mg *adriamycin bolus injection* (1 min) *in a leukemic patient.* ●: *Adriamycin concentrations in leukemic cells expressed in FU/cell (left) and in* ng/ml *(right) respectively, assuming* 10^9 *cells represent a volume of* 1 ml. ○: *Plasma concentration of adriamycin in ng/ml.* □: *Plasma concentration of the metabolite adriamycinol in* ng/ml.

911 ± 129 ng/ml for normal blast cells (Table 2). Cellular ADM concentrations in leukemic blast cells (n = 7) were in the same range as those obtained in normal bone marrow blast cells (n = 4), whether they were freshly obtained or thawed after cryopreservation (no significant differences). Inhibition of clonogenicity was in a similar range as well (Table 1).

Cellular ADM concentrations in vivo

An example of a plasma and cellular (blast cells) ADM concentration–time curve is shown in Fig. 3. ADM (30 mg/m²) was administered as a 1 min bolus injection. In the blast cells a short-lasting peak-cellular ADM concentration of 96 FU/cell (4160 ng/10^9 cells) was observed at 1.5 min after the start of the infusion, followed by a longer-lasting plateau concentration, starting at 10 min of approx. 60 FU/cell. At 24 h the cellular ADM concentration still amounted to 46 FU/cell (not shown in the figure).

After the three consecutive ADM injections, maximum cellular ADM concentrations at 5 min increased from 96 ± 29 FU/cell in circulating leukemic cells (n = 8 patients) to 216 ± 98 FU/cell on the 3rd day. These levels had decreased rapidly by 30–50% 1–2 h after the bolus injection. The apparently tightly bound cellular drug concentrations were 62 ± 20, 97 ± 43 and 122 ± 50 FU/cell respectively on days 1, 2 and 3 (Table 2). Similar values were observed in normal cells (n = 3 patients): 140 ± 69 FU/cell on day 1 decreased to 69 ± 21 FU/cell 1–2 h later. In contrast to the moderate decrease of cellular ADM concentrations, plasma concentrations were reduced by 2–3 decades, from maximum concentrations at 5 min of over 8000 ng/ml to 10–30 ng/ml after 2 h. Peak plasma adriamycinol concentrations were approx. 30 ng/ml at 1.5 min. Cellular adriamycinol was never observed, which indicated that, if it occured, it was below our detection limit of 1 ng/10^9 cells.

Clonogenicity and cellular ADM concentration in vivo (Table 3)

The range of tightly-bound cellular ADM concentrations *in vivo* is indicated in Fig. 2A. The highest inhibition of clonogenicity which can be expected from the *in vivo* observed cellular ADM concentrations is approx. 60–70% with this adriamycin treatment schedule.

In order to assess whether this extrapolation from *in vitro* data reflected the *in vivo* situation, inhibition of clonogenicity was determined after *in vivo* ADM exposure (Table 3). In two patients with normal bone marrow, clonogenicity was depressed to 79% and 44% respectively, with blast cell adriamycin concentrations of 53 and 74 FU/cell, at 1 h after 30 mg/m² bolus injection. In the third patient inhibition of clonogenicity was determined for bone marrow cells obtained before, at 5 min and 2 h after an ADM bolus injection of 50 mg/m². The number of colonies had been reduced to 13% and 19% respectively (blast cell ADM concentration: 144 and 71 FU/cell). The fourth patient was treated

Table 3. In vivo *observed inhibition of clonogenicity after adriamycin monotherapy*

Patient	Dose (mg/m²)	Before (No. of colonies)	Time after administration (% growth) 5 min	1 h	2 h	24 h	1 wk	Adriamycin concentration (FU/cell)
1*	30	96 ± 8		79 ± 4				53
2*	30	79 ± 6		44 ± 7				74
3†	50	140 ± 6	13 ± 4					144
					19 ± 5			71
4‡	40	107 ± 15	54 ± 6					69 ± 12
						37 ± 35		52 ± 21
							104 ± 8	0

*Normal bone marrow: acute nonlymphocytic leukemia in remission.
†Normal bone marrow: Hodgkin's disease, no bone marrow involvement.
‡Chronic myeloid leukemia in blast crisis.
Cells were cultured before and at indicated times after adriamycin monotherapy. Growth was expressed as % of the control (before adriamycin), set at 100%. Cellular adriamcyin concentration was expressed in arbitrary fluorescence units per cell (FU/cell).

with ADM infusions of 40 mg/m² every 2 months on three occasions. Inhibition of clonogenicity was assayed at the end of the infusions and some days thereafter. At maximum cellular drug levels of 69 ± 12 FU/cell, inhibition of clonogenicity was 54 ± 6% at the end of the infusion. This value was 37 ± 35% one day afterwards; despite persistence of some cellular ADM. One week later no inhibition was observed: clonogenicity was 104 ± 8% (Table 3).

DISCUSSION

One of the proposed modes of action of ADM is binding to DNA by intercalation between base pairs [14]. In cytotoxicity studies using cell lines, resistance to ADM was only marginally related to differences in the rate of uptake [15, 16], but closely related to the cellular retention of the drug.

In this study human hematopoietic clonogenic cells were exposed to ADM *in vitro* as well as *in vivo*. The purpose was to relate colony growth inhibition to ADM concentrations retained in the blast cells. Therefore, cellular drug concentrations were measured immediately after the washing procedure *in vitro*, or after the more than 2 log drop of the plasma ADM concentration *in vivo*. At longer periods thereafter an indication of certain repair was observed (Patient 4, at 24 h, Table 3), despite persistence of cellular ADM concentrations. This correlates with observations of long lasting tissue ADM concentrations on the one hand, and the clinical observations of the leucocyte nadir approx. 14 days after an ADM injection on the other hand (pointing to renewal of the proliferation at stem cell level approx. 10–14 days earlier, i.e. 1–4 days after the chemotherapy).

Flow cytometry enabled quantitative measurement of mean cellular ADM concentrations in very small numbers of viable cells of blast cell subpopulation [13, 17]. Whether this also reflects the concentration in the hematopoietic stem cell cannot be determined, since this cell cannot be identified clearly yet. The presence of other fluorescent metabolites *in vivo* or *in vitro* was excluded by determination of cellular drug concentrations by HPLC [6, 13]. A possible influence of non-fluorescent metabolites could not be ruled out.

In this small number of patients no significant differences in cellular ADM retention were noted among leukemic blast cells obtained from different patients, nor between leukemic and normal blast cells. The inhibition of clonogenicity was in a comparable range as well, for leukemic and normal bone marrow. This is in agreement with observations by Buick *et al.* [18], but in contrast with other studies [19, 20]. Of the seven patients, from whom marrow was used for *in vitro* incubations, complete remissions of various durations were obtained in four patients, two died during remission induction. One patient in this study appeared completely resistant to various remission induction courses (Fig. 2B). ADM uptake and retention by his blast cells did not differ essentially from those in the other patients or controls, neither *in vitro* nor *in vivo*. Such observations stress the need for further investigations of the role of cellular ADM concentrations and its related cytotoxic effect in clinical resistant cells *in vivo*, as opposed to the marked differences observed in cellular drug concentrations in sensitive and resistant cells *in vitro*.

A good correlation was observed between the cellular ADM concentration in leukemic and normal blast cells and inhibition of clonogenicity. However, the data do not permit the prediction of response to treatment outcome in leukemia, since such treatment always consists of other drugs as well. More than 2 log reduction, and near-complete inhibition, deduced from extrapolation of the rather steep growth inhibition curves, can only be expected after 2-h exposure to more than 2–4 μg/ml, or at

cellular ADM concentrations of over approx. 1200 FU/cell. This is equivalent to approx. 60 fg/cell, or 120×10^6 molecules ADM/cell. The number of ADM binding sites per cell has been calculated to be 1.5×10^9 [17]. Thus, an average of approximately 1:13 occupied binding sites produces substantial inhibition of clonogenicity.

The highest cellular ADM concentrations observed *in vivo* are far below the cellular drug concentrations, at which after *in vitro* exposure more than 2-log inhibition of clonogenicity was achieved [9, 20]. And, although the reduction of (leukemic) clonogenic cells is enhanced due to the usually polychemotherapeutic approach, in practice in most cases a relapse is observed after some time, which points to insufficiently high final drug concentrations in leukemic clonogenic cells. Also, a steep dose–response relationship was seen for the hematological toxicity and response rate [21, 22] in studies in which treatment of breast cancer patients with single agent ADM administration was investigated. Severe myelosuppression was noted at 3×45 mg/m^2 [21], a dose only 50% higher than used in our study. Since cellular and plasma ADM pharmacokinetics are linear [23], it can be extrapolated that the reported myelosuppression occured at cellular drug levels of approximately 180 ± 60 FU/cell (Fig. 2A), more on the steep part of the curve. However, administration of such higher dosages of ADM caused severe mucositis [21] and would kill a substantial number of normal clonogenic cells as well. Analogous to clinical observations, reporting a correlation between greater degrees of leukopenia and longer disease-free survival [21], our study provides some arguments for the fact that in leukemia residual disease may in part be due to limited cellular ADM concentrations attained with present-day therapeutic regimens. This stresses the point that in the treatment of leukemias, and probably in that of other malignant diseases as well, other approaches like ablative chemotherapy with bone marrow rescue should be investigated intensively.

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