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Comparative Studies on the in vitro Killing of Human Normal and Leukemic Clonogenic Cells (CFUc) by Daunorubicin, Daunorubicinol, and Daunorubicin-DNA Complex

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Summary. *The sensitivity of normal and acute nonlymphocytic leukemia-derived granulocytic stem cells (colony-forming units in culture, CFUc), exposed to daunorubicin, daunorubicinol, or daunorubicin-DNA complex in short-term suspension culture in vitro was studied. Normal CFUc were found to be considerably more sensitive to daunorubicin than to daunorubicinol. Killing of normal marrow CFUc by daunorubicin was an exponential function of the dose within the time and dose range tested. The C_{50} varied between 100 and 230 ng/ml medium, LD_{37} between 53 and 79 ng/ml medium. The response of CFUc to daunorubicin-DNA complex was exponential with somewhat larger individual variations (C_{50} = 100–330 ng/ml, LD_{37} = 46–878 ng/ml). The in vitro sensitivity of leukemia-patient derived CFUc to daunorubicin showed a greater variation (C_{50} = 127–454 ng/ml, LD_{37} = 74–640 ng/ml) than that of normal-control derived CFUc. In comparative studies with the leukemia-derived CFUc, daunorubicin-DNA complex was more or as effective as the free drug in killing CFUc derived from some patients, but in killing the CFUc derived from other patients was far less effective than the free drug. The results indicate greater individual variations in the response of leukemia-derived than normal CFUc both to daunorubicin and to daunorubicin-DNA complex in vitro. The therapeutic relevance of the results is discussed.*

Introduction

One of the important factors in the treatment of hematologic malignancies by cytostatic drugs is the relation between the effect of the drug on clonogenic malignant (e.g., leukemic) cells and on hematopoietic stem cells, both being responsible for the maintenance of respective cell populations. Cardiotoxicity aside, the myelosuppressive effect of daunorubicin is the side effect which limits the use of the drug in the treatment of malignant diseases. In an attempt to increase the selective action of daunorubicin on malignant cells, Trouet et al. (1972) exploited the elegant idea of lysosomotropism. By linking daunorubicin to a macromolecular carrier, e.g. deoxyribonucleic acid (DNA), daunorubicin was supposed to be taken up by endocytosis. It was speculated that a higher endocytic capacity of malignant cells would lead to a greater accumulation (and a greater cytotoxic effect) of the drug in these cells than in normal cells with lower levels of endocytosis. This theory also supposed that the target cell possesses enzymatic activity necessary for the release of drug from the carrier in the cell (Trouet et al., 1972).

Beside the comparative studies on toxicity and therapeutic effectiveness of daunorubicin and daunorubicin-DNA complex in animal models, which have been somewhat contradictory (Trouet et al., 1972; Henry, 1974; Ohnuma et al., 1975; Seeber et al., 1977), clinical trials with daunorubicin-DNA complex were started in patients with acute leukemias (Sokal et al., 1973; Cornu et al., 1974).

At present, however, no data is available concerning the influence of the linkage of daunorubicin to DNA on the toxicity of the drug to human hematopoietic stem cells and leukemic clonogenic cells.

The acute nonlymphocytic leukemia (ANLL) is a heterogenous disease assessed both morphologically (Flandrin and Bernard, 1975; Galton and Dacie, 1975) and by in vitro culture criteria (Moore et al., 1974; Spitzer et al., 1976; Hörnsten et al., 1977; Beran et al., 1979). It would therefore not be too surprising if the relationship between the endocytic ability of normal and leukemic cells varied from patient to patient. Recent advances in the in vitro growth techniques of human hematopoietic cells offer the unique opportunity of studying the response both of normal and of leukemic clono-

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genic cells to cytostatics in vitro. We have studied the quantitative aspects of the in vitro killing of normal and leukemia-derived colony-forming cells (CFUc) by daunorubicin and daunorubicin-DNA complex, as a part of a broader program on the therapeutic effectiveness of anthracycline glycosides. The main problems studied were as follows:

1. Kinetics of human CFUc killing by daunorubicin (D) and its main toxic metabolite daunorubicinol (DOH).

2. The influence of the binding of daunorubicin to DNA carrier (D-DNA) on the in vitro killing of normal and leukemic CFUc.

3. The interindividual variations in the drug toxicity on CFUc.

Materials and Methods

Source of CFUc. The basic hematologic data from leukemic patients studied are given in Table 1. For one patient with myelofibrosis (kk) from whom circulating CFUc were studied during a 2-year period, the range of hematologic data for this period is also given in Table 1. Nonleukemic subjects who served as controls were normal adult volunteers.

Cell Preparations. Bone marrow samples collected in glass tubes containing 50 IU of preservative-free heparin were centrifuged in narrow (5 mm inner diameter) tubes and marrow cells collected as buffy coat. After washing in McCoy's medium, the cells were used for in vitro studies.

Chemicals. Daunorubicin (D) and daunorubicinol (DOH) were obtained from Pharma-Rhodia, Stockholm, Sweden. The purity of the compounds was confirmed by reversed-phase liquid chromatography (Eksborg, 1978). The solutions were always freshly prepared in phosphate buffered saline (PBS, pH 7.2). DNA (herring sperm, Sigma, purity grade VII) was dissolved in 0.9% NaCl and the complex with DNA was prepared according to Trouet et al. (1972).

Experimental Design. Bone marrow cells (4×10^6/ml in serum-free PBS) were exposed to different concentrations of each compound. The final concentrations were chosen to approximate those found in plasma during and immediately after infusion of D or its DNA complex in vivo (Eksborg et al., 1978; Andersson et al., 1978), i.e. 100–600 ng/ml. Higher concentrations were tested in experiments with leukemic cells from therapy-resistant leukemic patients. Cells incubated in PBS only served as controls. In most experiments, cells were incubated with drug for 1 h at 37° C. Time response studies were performed during 1–4 h.

After incubation, the cells were washed twice in 10 ml PBS (10 min centrifugation at 400 *g* after each washing) to eliminate the extracellular drug and resuspended in 0.5–1.0 ml Hepes buffered McCoy's medium. Different numbers of cells were mixed with agar medium and the number of surviving CFUc was assayed by the modified Robinson's culture technique (Beran et al., 1979). Cultures were incubated for 8–10 days at 37° C in a fully humidified atmosphere of 5% CO_2 in air.

Culture Evaluation. At the end of the culture period, clones of cells arising by proliferation of CFUc were counted. For the purpose of comparison with leukemic cultures which differ in the growth pattern from the growth of normal CFUc (Beran et al., 1979) and in which only small clones containing often less than 50 poorly differentiated cells are formed, clones containing more than eight cells were counted both in normal and in leukemic cultures. This characteristic growth is taken as evidence of malignant population (Moore et al., 1974; Spitzer et al., 1976; Beran et al., 1978). Hence, survival studies were based on clones containing more than eight cells. It should be noted that the 'leukemic' growth pattern (Beran et al., 1978) can be obtained in only approximately one-third of patients with ANLL. The cells in cultures from the remaining two-thirds of patients either do not grow at all or only a poor growth is obtained, characterized by few clones of 'normal' size probably arising from the persisting nonleukemic CFUc. These were not included in the present study.

The surviving fraction for each drug concentration was calculated by comparing the number of CFUc after drug exposition to that in control cultures. Survival curves were constructed and the C_{50} (concentration of the drug in medium which kills 50% of CFUc) and LD_{37} (dose of the drug required to reduce surviving fraction of CFUc to a value of $1/e$, e being the exponential function and $1/e$ equals 0.37) were calculated. Using LD_{37}, which characterizes slopes of survival curves, these for CFUc obtained from different patients can be compared.

Results

Response of human CFUc treated in vitro with varying doses of either D or its metabolite DOH is shown in Fig. 1. The survival curves for both D and DOH are

Table 1. Basic hematologic data on leukemia patients and a patient with myelofibrosis

Patient	Sex	Age (years)	Diagnosis	Total WBC (1×10^9/l)	Blast cells (%)	Platelets (1×10^9/l)	Hb (g/l)	Cytostatics before testing
jb	M	77	AUL	34	22	10	82	no
md	F	64	AML	28	3	30	119	no
kl	F	37	AUL	91	40	21	83	yes
th	M	69	AMML	33	30	61	88	yes
gd	M	75	SL	7	1	53	49	no
jk	F	65	APmL	55	14	132	59	no
kk	M	85	Mf	17–66	0–1	175–83	71–94	no

AUL = acute undifferentiated leukemia; APmL = acute promyelocytic leukemia; AMML = acute myelomonocytic leukemia; AML = acute myeloblastic leukemia; SL = smoldering leukemia (terminal phase); Mf = myelofibrosis; M = male; F = female

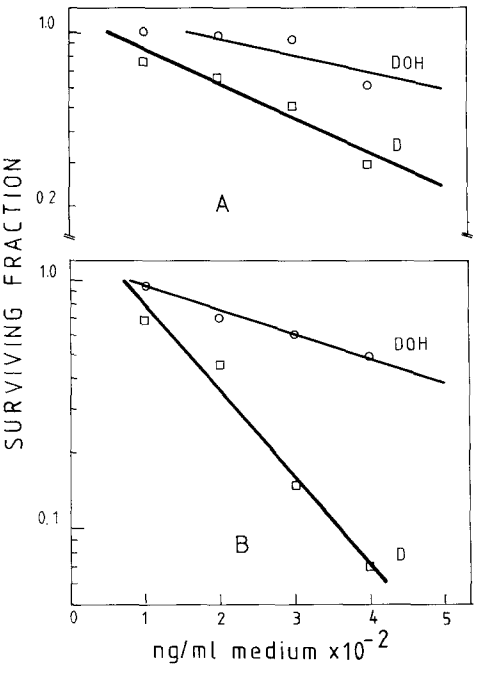

Fig. 1. The response of human CFUc exposed in vitro for 60 min to varying doses of either daunorubicin (*D*) and daunorubicinol (*DOH*). The response of circulating CFUc from a patient with myelofibrosis is shown in **A**, and **B** shows the response of CFUc from bone marrow of a representative normal subject. In a patient with myelofibrosis (**A**) the C_{50} value was 600 ng/ml for DOH and 266 ng/ml for daunorubicin; the LD_{37} values were 639 ng/ml and 304 ng/ml, respectively. For normal marrow CFUc (**B**) the C_{50} values were 382 ng/ml for DOH and 153 ng/ml for daunorubicin; the LD_{37} values were 442 ng/ml and 125 ng/ml of DOH and daunorubicin, respectively

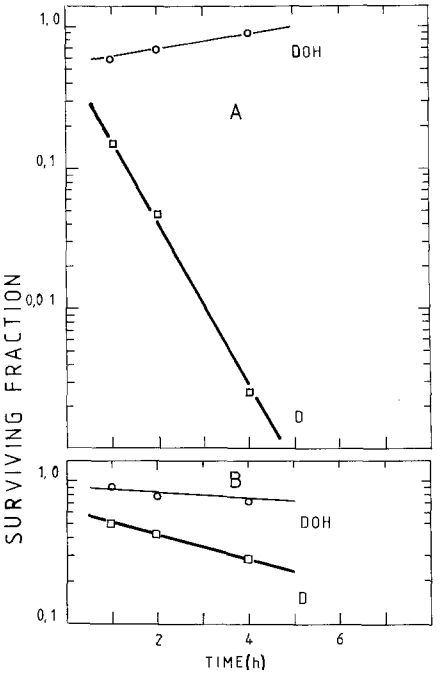

Fig. 2. Time response of bone marrow CFUc from a representative normal subject (**A**), and of circulating CFUc from a patient with myelofibrosis (**B**). Cells were exposed simultaneously to 300 ng/ml of daunorubicin (*D*) or daunorubicinol (*DOH*) in PBS

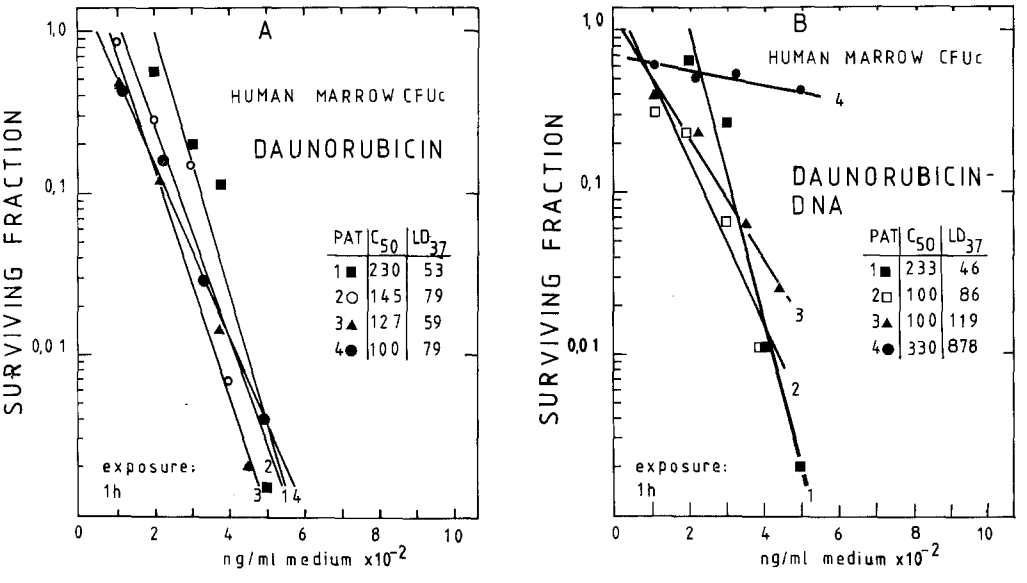

Fig. 3. The in vitro response of normal human bone marrow CFUc exposed in vitro to varying doses of daunorubicin (**A**) or daunorubicin-DNA complex (**B**). The *numbers* refer to identical donors of bone marrow (*1–4*). Tables in **A** and **B** show D_{50} and LD_{37} values for CFUc from individual donors

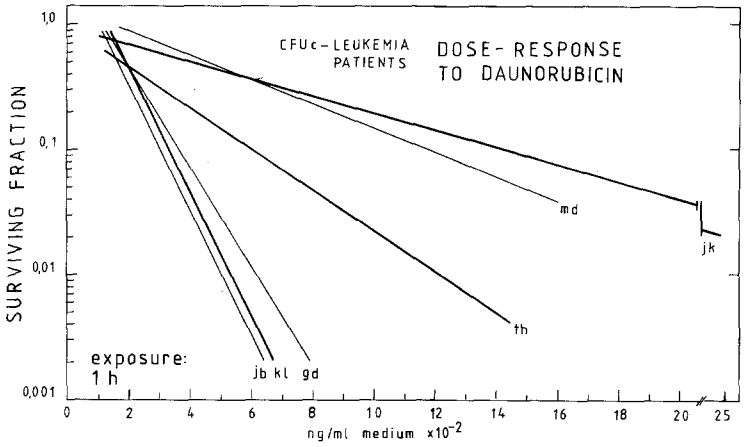

Fig. 4. Dose-response curves of leukemic CFUc from patients with ANLL to daunorubicin. In two patients (*th* and *jk*) with acute promyelocytic and myeloblastic leukemia, respectively, the CFUc were tested in the terminal stage of the disease, when the patients were resistant to daunorubicin treatment. One patient (*kl*) was previously treated with cytostatics other than anthracyclines. CFUc from remaining three patients were tested before any cytostatics were given. Note the 'resistant' slope of the *survival curve* from a previously nontreated patient (*md*)

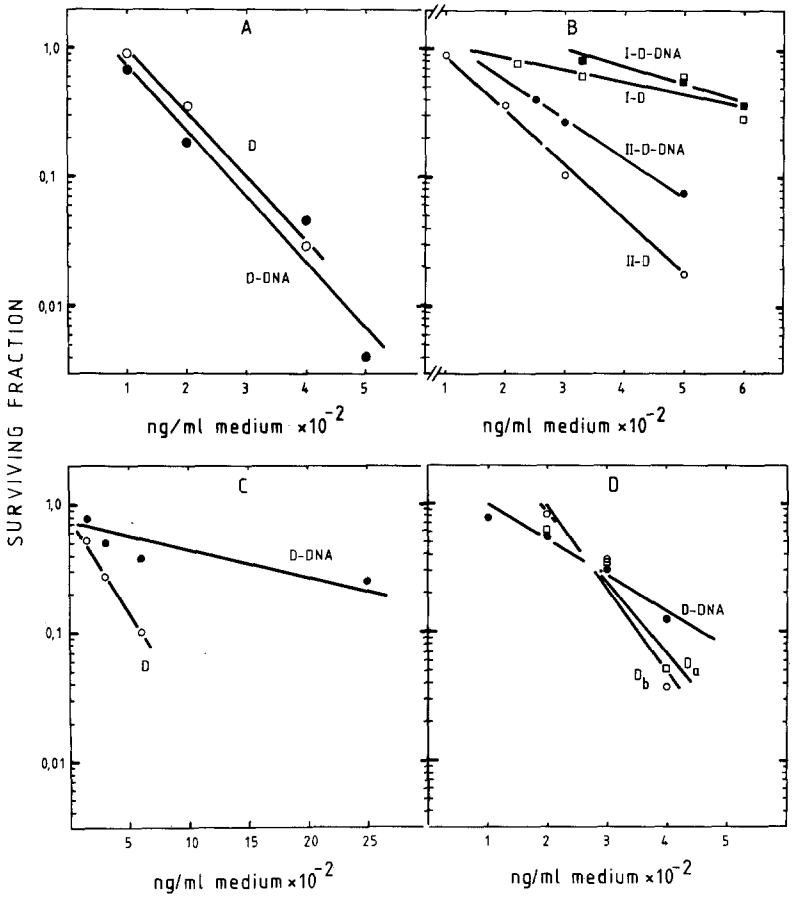

Fig. 5. Comparative studies on the response of leukemic CFUc to daunorubicin (*D*) and daunorubicin-DNA complex (*D-DNA*) in vitro. Cells from each patient were simultaneously exposed to varying doses of respective compound for 1 h in vitro. Note the variation in the sensitivity to the daunorubicin-DNA in comparison to free drug. The higher sensitivity of CFUc to daunorubicin-DNA in **A** contrasts with far lower sensitivity of CFUc from a patient in **C**. *Roman numerals* in **B** refer to two different patients (*I* = md, *II* = kl). Symbols *a* and *b* in **D** (patient gd) refer to survival curves obtained after exposing cells from identical marrow samples to daunorubicin in two independent parallel experiments, and indicate the reproducibility of the used technique. *Survival curves* in **A** refer to CFUc from patient jb, and in **C** from patient th.

essentially exponential over the dose range tested. DOH was far less toxic in vitro than D, both to circulating CFUc from a patient with myelofibrosis (Fig. 1A) and to bone marrow CFUc from a representative normal subject (Fig. 1B). Whereas the killing of normal CFUc by D increased with the exposure length (Fig. 2A), no significantly increased killing by DOH was noted on prolonged exposure. Similar results were obtained with CFUc from the blood of a patient with myelofibrosis (Fig. 2B), although on repeated examinations (not shown here) the CFUc from this patient were constantly less sensitive to D than CFUc from marrow of normal subjects.

Figure 3 shows the results of comparative studies on the response to graded doses of D or D-DNA complex of marrow CFUc from four normal subjects. Identical

marrow samples from each subject were exposed to cytostatics concomitantly. The results show that CFUc are killed by both preparations of the drug in an exponential fashion with minor interindividual variation in the sensitivity to the D (C_{50} = 100–230 ng/ml medium). The response to D-DNA complex was not very different with respect to C_{50} (100–330 ng/ml medium). The slopes of the survival curves (LD_{37}) were, however, far more variable for D-DNA complex with a tendency to proportionally lower kill with increased dose as compared with free drug. Also, the interindividual variations were larger for the D-DNA complex.

Response of Human Leukemic CFUc to Daunorubicin

Like the response of normal CFUc (Fig. 3A), that of leukemic CFUc was an exponential function of the D dose (Fig. 4). The results clearly demonstrated a much larger variability in the drug sensitivity of leukemic than normal CFUc in vitro. Both C_{50} (127–388 ng/ml) and particularly the slopes of survival curves (LD_{37} = 73–792 ng/ml) varied widely between CFUc from various leukemic patients. Importantly, the in vitro-resistant CFUc (patients th and jk, Fig. 4) were derived from patients at the terminal stage of the disease, completely resistant to D treatment.

Comparative Response of Human Leukemic CFUc to Daunorubicin and Daunorubicin-DNA

In five patients with ANLL, the response of leukemic CFUc to increasing doses of D and D-DNA complex was studied simultaneously in vitro. The results summarized in Fig. 5A–D show variations in the relative response of leukemic CFUc to D-DNA complex as compared with free drug. Leukemic CFUc from some patients responded to both forms of treatment by almost identical survival curves (Fig. 5A and B). In others, however, D-DNA complex proved less effective in killing the leukemic CFUc in vitro (Fig. 5C–D) than did the free drug.

Discussion

An in vitro test system using the reproductive integrity of cells as end point has been used in this study to evaluate the toxic effect of D, DOH, and D-DNA complex. In this way only clonogenic cells, represented by normal granulocytic stem cells or leukemic stem cells, both responsible for further maintenance of respective cell lines, have been assayed.

In our assay system, the D metabolite DOH was significantly less toxic to human CFUc in vitro compared with parent compound. This agrees with what Asbell et al. (1972) found in growth inhibition assay with L 1210 cells. However, the toxicity of D in vivo against P 388 cells growing in mice was by these authors (1972) found to be equivalent to that of DOH. Whereas CFUc inactivation by D in vitro increased as a function of the time, a prolonged exposure of identical cells to DOH did not significantly decrease the surviving fraction. Whether these differences depend on the uptake mechanism or intracellular events cannot be concluded from our results.

In contrast to rather small interindividual variations in the sensitivity of normal marrow CFUc to D, the sensitivity of 'leukemic' CFUc from different patients with ANLL varied within a wide range. Whether these variations have clinical relevance remains to be determined, but in two patients the increased in vitro resistance of leukemic CFUc to D correlated well with the total resistance of leukemic population to treatment of the patients with this drug. In these patients, the resistance could have been due to the selection of a resistent clone during the treatment. It appears, however, that a high resistance in vitro is also an inherent property of CFUc derived from some nonleukemic as well as leukemic subjects. This is supported by the finding that CFUc from one patient with previously untreated leukemia (Fig. 4, md) were extremely resistant to D despite the lack of their previous contact with this drug in vivo. Similarly, in a patient with myelofibrosis, circulating CFUc were tested repeatedly during a 2-year period and despite some variations they were always much less sensitive to D than were normal marrow CFUc. In this patient, the S-phase fraction of circulating CFUc was comparable to that in normal bone marrow, thus the differences in sensitivity are not explainable by the differences in the proliferative state of CFUc populations (unpublished data).

Compared with the in vitro response of normal CFUc to free drug, the response of identical cells to D-DNA complex varied slightly more. In one subject, the CFUc were highly resistant to the DNA-complexed D but had ordinary sensitivity to free drug. The differences in sensitivity of the CFUc population from identical subjects to D and D-DNA complex apparently do not depend on the test technique. Repeated in vitro sensitivity testing of CFUc from one subject over a period of two years always gave similar results with respect to relative sensitivity to free and DNA-complexed drug.

Our present findings might have relevance in selecting the individual treatment schedules for patients with ANLL. At least in some patients the variations in the in vitro resistance to D-DNA complex could reflect the inherent and/or acquired property of the leukemic clo-

nogenic cells (uptake mechanism? intracellular processing of te DNA complex?) which render them more resistant to DNA complex than are normal CFUc. In such patients the use of DNA complex would be less effective in treatment of the leukemia despite some benefit in the form of (hopefully) decreased cardiac toxicity.

The presented results demonstrate an appreciable heterogeneity in the sensitivity of leukemic clonogenic cells (CFUc), both to D and particularly to its complex with DNA. In vivo, the response of cells is further influenced by a number of variables such as the pharmacokinetics of the drug, its accumulation in the tissues, and, in the case of D-DNA complex, its stability in the circulation, which determines the amount of drug taken up by cells as complex with DNA and as released free drug. With this in mind, the presented results have to be considered as one step toward a better understanding of the interaction between cytostatics and human normal and leukemic cells. The relevance of these in vitro findings is presently being investigated in leukemic patients in vivo.

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