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Cytotoxicity of Doxorubicin for Normal Hematopoietic and Acute Myeloid Leukemia Cells of the Rat

P. Sonneveld[1], J. A. Mulder[2], and D. W. van Bekkum[1]

[1] Radiobiological Institute TNO,
P.O. Box 5815, 2280 HV Rijswijk
[2] Delft University of Technical Sciences, The Netherlands

Summary. *The dose response curves of doxorubicin for hematopoietic rat bone marrow cells were investigated and compared with the dose-response curves of doxorubicin for leukemia cells from bone marrow and spleen of rats inoculated with an acute myelocytic leukemia (BNML). Various assays were used to determine the cytotoxicity of doxorubicin. It was found that the inhibition of DNA synthesis by doxorubicin compared well with results obtained with in vivo assays for the determination of clonogenic hematopoietic (CFU-S) and leukemic (LCFU-S) cells. It was found that doxorubicin at concentrations ranging from* $0.1-1.0\,\mu g \cdot 10^{-7}$ *cells inhibits DNA synthesis of leukemic cells to 60% and that of hematopoietic cells to 90%. Higher doxorubicin concentrations further inhibit DNA synthesis of only hematopoietic cells. These results were confirmed with clonogenic assays. Pretreatment with the S phase-specific drug arabinoside cytosine (ara-C) increased the efficacy of doxorubicin in vitro significantly. In view of the doxorubicin concentrations in bone marrow obtained in vivo* ($\geq 1\,\mu g \cdot 10^{-7}$ *cells), it is concluded that dosage reduction may reduce toxicity with no concomitant decrease of antileukemic activity of doxorubicin.*

Introduction

Doxorubicin (adriamycin) is widely used in the treatment of acute myelocytic leukemia (AML) [6]. However, its therapeutic effect is often complicated by the serious bone marrow toxicity [6].

There is presently a serious lack of literature data on the comparative sensitivity of hematopoietic and leukemic cells to the cytotoxic action of doxorubicin, but certain differences, if present, might be relevant for the determination of the drug dose in vivo [13].

Reprint requests should be addressed to: P. Sonneveld

Recently, Buick et al. [4] published some data on anthracycline cytotoxicity to plated normal and leukemic blast cells. They showed that negative exponential dose-response curves were obtained when cells were cloned after doxorubicin exposure. However, a great variability could be detected in the D_{10} stands for: doses at which 10% of the cells are killed of normal and leukemic cells.

Therefore, the present studies were performed to investigate the comparative sensitivity to doxorubicin in vitro of normal bone marrow cells and leukemic cells obtained from rats inoculated with the Brown Norway Myelocytic Leukemia (BNML). This leukemia is characterized by a slow net growth rate, early suppression of normal hematopoiesis, and a response to chemotherapy that is identical to that in patients with AML [5]. Since, in the case of full-blown leukemia, both normal hematopoietic and leukemic cells lodge and proliferate in bone marrow and spleen, cells isolated from these organs were used to investigate the differential effects of doxorubicin on normal bone marrow cells and BNML cells. The following experimental issues were investigated in vitro:

a) The uptake and elimination of doxorubicin in vitro;

b) Determination of the ratio of dead/viable cells as a function of the concentration of doxorubicin in the in vitro incubation medium by means of the fluorescence light-scatter characteristics of the cells;

c) The effect of doxorubicin on DNA and RNA synthesis;

d) Doxorubicin-induced effects on the proliferative state of the cells in vitro;

e) The effect of doxorubicin on clonogenicity of cells.

At present, these in vitro phenomena offer the best means of monitoring the effect of cytotoxic drugs

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in those tumor types that cannot be cultured in vitro [9].

Materials and Methods

Animals

Female rats of the BN inbred strain, raised and bred under specific pathogen-free (SPF) conditions, were used. The animals were fed standard AM pellets (Hope Farms, The Netherlands) and had free access to water.

Leukemia

Spleen cells of leukemic animals were suspended in Hanks' balanced salt solution (HBSS) and 10^7 leukemic cells were transplanted intravenously in a volume of 1 ml. After transplantation the survival ranged from 22–24 days. For all experiments leukemic cells of either femoral or splenic origin were used, obtained from animals at day 15 of the disease. When liver and spleen are enlarged, the bone marrow contains more than 90% of leukemic cells, and leukemic cells start to appear in the peripheral blood. At this stage the disease is comparable with that at the time of clinical diagnosis in man.

Chemicals, Media, and Labeling Conditions

Doxorubicin was a gift from Farmitalia, Milan, Italy. Fresh solutions were prepared before each experiment. The incubation medium used was always HBSS. ^{3}H-Thymidine (^{3}H-TdR; specific activity, 2–5 Ci · mMol^{-1}) and ^{3}H-Uridine (^{3}H-UdR; specific activity, 20–40 Ci · mMol^{-1}) were purchased from the Radiochemical Centre, Amersham, England. Radioactivity of the ^{3}H-labeled compounds was measured by liquid scintillation counting (Nuclear Chicago, Mark II counter). Labeled cells were harvested on glassfiber filters (type A–E, Gelman, Ann Arbor, Mich., USA). The air-dried filters were placed in scintillation vials (Packard Instruments, Zurich, Switzerland) and 2 ml toluene-based scintillation fluid (50 mg POPOP and 4 g PPO per liter toluene) was added.

DNA and RNA Synthesis in Spleen and Bone Marrow Cells

Normal and leukemic animals were anesthesized with ether and killed by cervical dislocation; spleens were removed, weighed and collected in HBSS. The femurs were removed, cut, and also placed in HBSS. Spleens were finely minced separately with a pair of scissors and were filtered repeatedly through six layers of nylon gauze. The bone marrow cells were collected by repeatedly flushing the femoral shaft with HBSS. The collected bone marrow was filtered through six layers of nylon gauze and suspended with HBSS to a final concentration of 10^7 cells · ml^{-1}. Doxorubicin dissolved in saline was added at different concentrations in a final volume of 0.1 ml and the cells were incubated in a water bath at 37° C in closed tubes for 1 h. Then, ^{3}H-TdR at a dose of 1 μCi · 10^{-7} cells or ^{3}H-UdR at a dose of 1.5 μCi · 10^{-7} cells was added in a final volume of 0.1 ml; the tubes were shaken and incubated again for 30 min. After this period, the incorporation was terminated by adding an ice-cold solution of unlabeled thymidine in saline. After repeated washing of the cells with saline, they were harvested on glassfiber filters. Results were expressed as the absolute number of counts per minute (cpm) of triplicate samples.

Cellular Influx and Efflux of Doxorubicin

Animals were anesthesized with ether and killed by cervical dislocation. Bone marrow cells were suspended in HBSS in a volume of 10^8 per ml. Doxorubicin was added at various concentrations in a final volume of 0.1 ml and the cells were incubated at 37° C for 1 h in a water bath. After incubation, the cells were washed twice and either placed at 4° C or reincubated in doxorubicin-free medium. These reincubated cells were washed afterwards with saline and placed on ice. The intracellular content of doxorubicin was determined spectrofluorometrically. After drug extraction from the cells according to the method described by Schwartz [8] the relative fluorescence, obtained by comparison with known standards, was used to determine the intracellular drug concentrations.

Assay for the Quantification of Leukemic and Hematopoietic Clonogenic Cells: Leukemic Colony-forming Unit, Spleen (LCFU-S) and Colony-forming Unit, Spleen (CFU-S)

Low numbers of BNML cells (5×10^3 to 5×10^4) injected intravenously into nonirradiated recipient BN rats grow out into colonies which can be counted on the surface of the spleen at day 19 after injection. A linear relationship has been found between the number of cells injected and the number of spleen colonies [12]. The leukemic origin of the colonies has been confirmed by injecting cells obtained from these nodules into secondary recipients. In the present study, BNML bone marrow cells treated with doxorubicin at various concentrations for 1 h in vitro were injected into BN recipients at concentrations varying from 10^3–10^6 per recipient and the number of spleen colonies was counted at day 19.

The number of pluripotent hematopoietic stem cells (HSC) in the BN rat can be determined with the modified CFU-S assay [9, 11, 12]. With this technique, bone marrow cells are injected intravenously into lethally irradiated mice. The stem cells that give rise to spleen colonies which can be counted at day 9 after injection are defined as colony-forming units, spleen (CFU-S). A linear relationship is found between the number of injected bone marrow cells and the number of spleen colonies in two recipient species: the BN rat and F1 hybrids of CBA × C57BL mouse strains.

In this study, normal bone marrow cells were incubated in vitro with various concentrations of doxorubicin for 1 h, and injected into F1 hybrid mice of C57BL/LiRij × C3H/LwRij. Nine days after injection of the cell suspension, colonies on the surface of the mouse spleen were counted after fixation of the spleen in Tellyesniczky's solution. The aim of this study was to investigate whether these clonogenic assays produce results comparable to those from the DNA and RNA inhibition assays, which, in fact, measure loss of cell function rather than loss of cell clonogenicity.

Results

When the effect of doxorubicin on various parameters of cell viability is determined, some quantitative

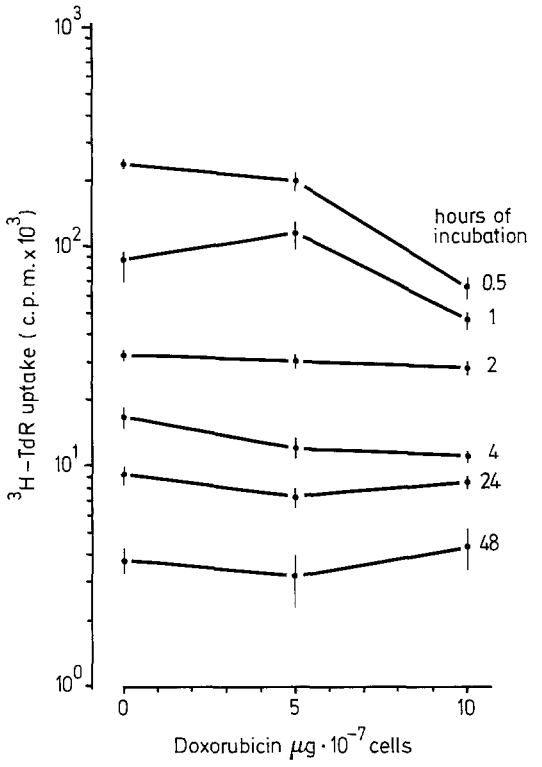

Fig. 1. Incorporation of ^{3}H-TdR in leukemic bone marrow cells after various periods of incubation with different concentrations of doxorubicin. Results are expressed as radioactivity present in the cells and represent the mean ± 2 SE of five experiments

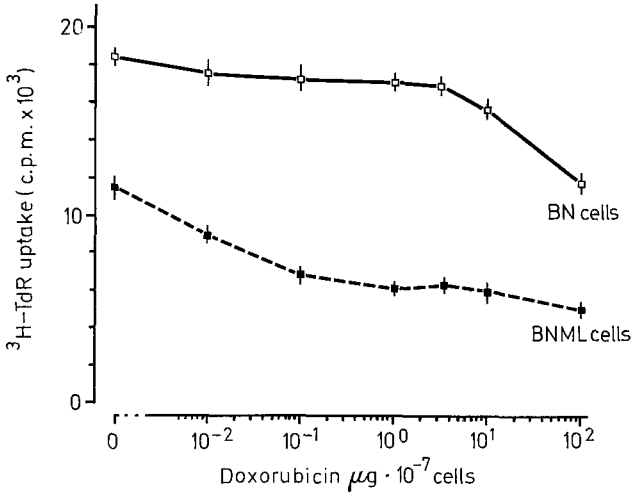

Fig. 2. Incorporation of ^{3}H-TdR in BN and BNML femoral bone marrow cells following 1 h of incubation with doxorubicin in vitro (M ± 2 SE of five experiments)

differences can be observed. For example, the inhibition of DNA synthesis as a function of the concentration of doxorubicin is significantly influenced by the duration of the incubation period. In these experiments, leukemic bone marrow cells were incubated with various concentrations of doxorubicin for periods ranging from 30 min to 48 h at 37° C. Thereafter, cells were washed, ^{3}H-TdR was added, and the cells were further treated as described before. During the incubation, the stability of the osmolarity and pH of the suspension was monitored. It appeared that no significant variations in osmolarity or pH occurred during incubation periods of up to 2 h. As shown in Fig. 1, the uptake of ^{3}H-TdR in the suspended cells is inversely related to the duration of the incubation period.

After periods of incubation longer than 2 h hardly any effect of doxorubicin on the uptake of ^{3}H-TdR can be demonstrated, while shorter periods lead to a concentration-dependent inhibition of the ^{3}H-TdR uptake. It can be concluded that incubation periods of 30 min to 1 h provide the optimal conditions for measuring the doxorubicin-induced inhibition of DNA synthesis. For incubation periods of 1 h, a clear concentration-dependent inhibition is found; therefore in further experiments this period was used (Fig. 2).

In Table 1, the doxorubicin-induced inhibition of DNA synthesis in leukemic cells is compared with its effect on the viability of BNML cells and their clonogenicity. For viability measurements the eosin staining method was used, while the LCFU-S assay was employed to determine the reduction of clonogenic leukemia cells after doxorubicin treatment. The reduction of clonogenic leukemia cells compares well with the inhibition of DNA synthesis at drug concentrations of 10 $\mu g \cdot 10^{-7}$ cells, while the number of dead cells, determined with eosin uptake, is not altered by doxorubicin. This finding implies that data about DNA synthesis apparently underestimate the doxorubicin-induced kill of clonogenic leukemic cells. This is probably because the LCFU-S assay reflects only clonogenic leukemic cell kill, while the ^{3}H-TdR uptake of all cells is measured. Therefore, the measurement of DNA synthesis may continuously be used to observe the effect of doxorubicin on BNML cells. In Table 2 the reduction of clonogenic leukemic cells and clonogenic hematopoietic cells is compared. It appears that the reduction of CFU-S at the two lower concentrations of doxorubicin is less than that of LCFU-S. This difference is reflected by the inhibition of DNA synthesis of leukemic and normal bone marrow cells.

Incubation of BN and BNML fermoral bone marrow cells for 60 min in the presence of different concentrations of doxorubicin leads to an intracellular accumulation of the drug, which is dependent on the concentration in the medium. As shown in Table 3, isolated BNML cells take up significantly more doxorubicin than do BN cells at drug concentrations

Table 1. Effect of doxorubicin on BNML bone marrow cells in vitro[a]

	Percentage of dead cells (eosin method) (M ± SD)	Reduction of DNA synthesis (M ± SD)	Reduction of LCFU-S (M ± SD)
Doxorubicin 0.1 μg · 10^{-7} cells	30.8 ± 5.6	29.6 ± 4.4	25.0 ± 10.1
Doxorubicin 1.0 μg · 10^{-7} cells	31.5 ± 3.4	41.7 ± 6.5	47.8 ± 24.3
Doxorubicin 10.0 μg · 10^{-7} cells	29.4 ± 4.3	48.2 ± 6.4	96.2 ± 13.6

[a] Expressed as percentage of saline-treated controls

Table 2. Number of LCFU-S and CFU-S after treatment with doxorubicin in vitro (percentage of controls)

	LCFU-S		CFU-S
Saline	100		100
Doxorubicin 0.1 μg · 10^{-7} cells	75 ± 10.8	$P < 0.05$	95 ± 8.7
Doxorubicin 1.0 μg · 10^{-7} cells	53.2 ± 19.7	NS	67.6 ± 14.5
Doxorubicin 10.0 μg · 10^{-7} cells	3.8 ± 2.7	NS	4.5 ± 1.1

NS, not significant

Table 3. Uptake and intracellular retention of doxorubicin in BN and BNML cells[a]

Extracellular concentration of doxorubicin μg · 10^{-7} cells	BN		BNML	
	Uptake	Retention	Uptake	Retention
0	0	0	0	0
1	0.4	0.2	0.8	0.5
10	3.2	2.7	5.0	4.4
50	7.7	6.1	9.5	8.8
100	9.4	8.3	13.2	12.0

[a] Expressed as μg · 10^7 cells

of 10 μg or more per 10^7 cells ($P < 0.001$). The two cell types do not differ in their capacity to retain the intracellular amount of drug when placed in a drug-free incubation medium. The remaining fluorescence in leukemic cells exceeds that in normal bone marrow.

The inhibition of ^{3}H-TdR incorporation as a function of the concentration of doxorubicin present was employed to investigate the dose-effect relation between doxorubicin and both normal hematopoietic and leukemic cells in vitro. When bone marrow cells of BN or BNML origin are incubated in the presence of various concentrations of doxorubicin and ^{3}H-TdR is subsequently added, the uptake of the latter compound is inhibited to a different extent in the two cell types. Low concentrations of doxorubicin (0.01–1.0 μg · 10^{-7} cells) lead to a decreased uptake of ^{3}H-TdR in BNML cells, while a high concentration (100 μg · 10^{-7} cells) inhibits the uptake of ^{3}H-TdR to 67% in BN and to 35% in BNML cells (Fig. 2). The control numerical values for the uptake of ^{3}H-TdR in BN cells are twice those in BNML bone marrow cells. This could be due to a relatively higher percentage of BN cells being in the S phase of the cell cycle. According to Hagenbeek [5], the labeling index (representing the percentage of cells in the S phase) is 58% for normal rat myeloblasts and 30%–43% for BNML cells, which findings may account for the observed difference in ^{3}H-TdR uptake in control samples. From the effect of doxorubicin on ^{3}H-TdR uptake, it may be concluded that a difference is demonstrable with respect to the sensitivity of BN

and BNML bone marrow cells. In BNML spleen cells (Fig. 3), low concentrations of doxorubicin ($0.01-0.1\ \mu g \cdot ml^{-1}$) inhibit the uptake of ^{3}H-TdR to 70% without any further decrease at higher drug concentrations. This inhibition is less than in BNML bone marrow cells, which may be explained by the fact that the labeling index of BNML spleen cells is higher than that of BNML bone marrow cells (0.48 versus 0.36).

Although the cytotoxic effect of doxorubicin has not been reported to be specific for one cell cycle phase [2, 3], its activity is more pronounced in cells which have been synchronized with arabinoside cytosine (ara-C) [1]. It is known that an appropriate dose of ara-C in vivo leads to recruitment of nonproliferating BNML cells into cycle with an increased (up to 60%) number of cells in the DNA synthesis phase of the cell cycle at 12 h following injection of ara-C [1, 2]. This phenomenon was used to investigate the action of doxorubicin in vitro on synchronized cells. For this purpose, bone marrow and spleen cells were obtained from normal and BNML rats which had received injections of ara-C ($200\ mg \cdot kg^{-1}$ i.v.) 12 h earlier. Figure 4 shows the in vitro effect of doxorubicin on BNML and BN bone marrow and spleen cells treated with ara-C in vivo. Addition of doxorubicin leads to a concentration-dependent reduction in ^{3}H-TdR uptake in these cells down to 10% of the control values at the highest concentration of doxorubicin ($10\ \mu g \cdot 10^{-7}$ cells). The same observation is made in suspended femoral BN cells. In all these cell types, the control ^{3}H-TdR uptake is twice that obtained with comparable cells, not pretreated with ara-C, except in BN spleen cells which are mainly lymphocytes.

Apparently, pretreatment with ara-C increases the sensitivity of nonexponentially proliferating cell populations to the action of doxorubicin, due to the greater number of cells in the S phase of the cell cycle.

The effect of doxorubicin on RNA synthesis as reflected by its effect on the uptake of ^{3}H-UdR into BN and BNML femoral bone marrow cells is presented in Fig. 5. As with DNA synthesis, a concentration-dependent reduction in the ^{3}H-UdR uptake is observed, which is more pronounced in BNML cells than in BN cells. The uptake of ^{3}H-UdR in leukemic cells is completely inhibited at the highest concentration of doxorubicin ($50\ \mu g \cdot ml^{-1}$), which

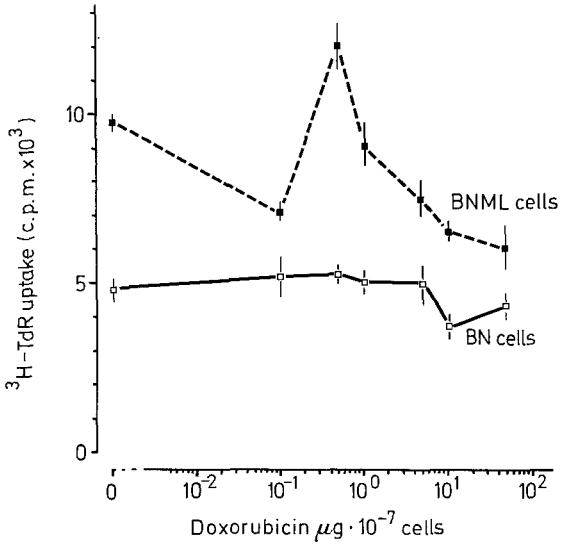

Fig. 3. Incorporation of ^{3}H-TdR in BN and BNML spleen cells following 1 h of incubation with doxorubicin in vitro (M ± 2 SE of five experiments)

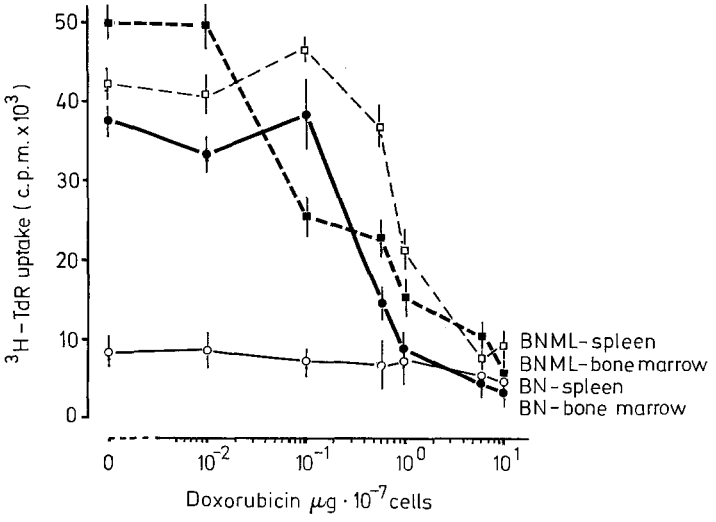

Fig. 4. Incorporation of ^{3}H-TdR in BN and BNML femoral bone marrow and spleen cells pretreated with ara-C in vivo following 1 h of incubation with doxorubicin in vitro (M ± 2 SE of three experiments)

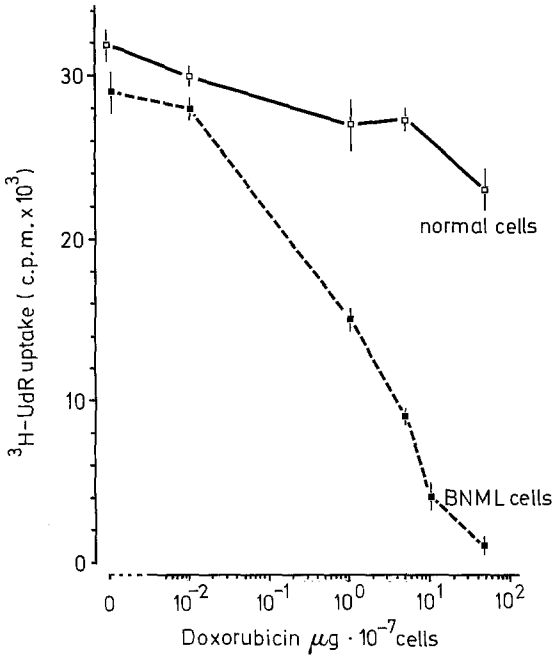

Fig. 5. Incorporation of ^{3}H-UdR in BN and BNML femoral bone marrow cells following 1 h of incubation with doxorubicin in vitro (M ± 2 SE of five experiments)

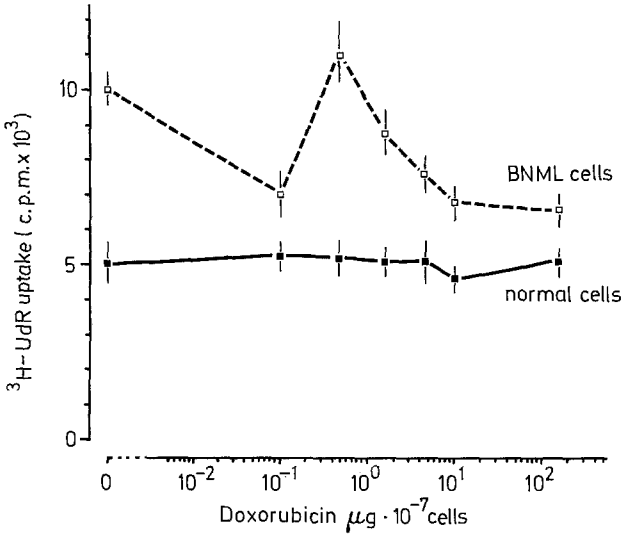

Fig. 6. Incorporation of ^{3}H-UdR in BN and BNML spleen cells following 1 h of incubation with doxorubicin in vitro (M ± 2 SE of five experiments)

suggests that RNA polymerase is blocked by doxorubicin to a greater extent in these cells [14]. The inhibitory effect of doxorubicin on suspended splenic BNML cells is less impressive, although comparable with its effect on ^{3}H-TdR uptake in these cells (Fig. 6). Normal spleen cells are not sensitive to doxorubicin in this respect.

Discussion

It has been suggested [7] that only the reduction in clonogenic cells in vitro gives a reliable indication of the response to chemotherapy in vivo. Although this statement may certainly be valid for those cells that can be cultured in vitro, it is not valuable to quantify antitumor effects of cytostatic drugs in vitro in those cells which cannot be cultured, such as in this BNML leukemia and most human acute leukemias. Therefore, in the present study, the relatively less valid assay for DNA synthesis inhibition has been compared with in vivo clonogenic assays for leukemic and hematopoietic cells, which were previously shown to yield reliable data on the reduction in blast cells following chemotherapy. With this rat leukemia, LCFU-S data can well be compared with data concerning the inhibition of DNA synthesis, but not with data obtained with the eosin staining method, which have proved to be inaccurate. Furthermore, LCFU-S and CFU-S data agree with data concerning the inhibition of DNA synthesis of leukemic and normal bone marrow cells, respectively. One restriction in the interpretation of data from the DNA synthesis is that at high concentrations of doxorubicin ($\geq$ 10 μg · 10^{-7} cells) the inhibition of ^{3}H-TdR uptake is not complete, while the reduction in LCFU-S and CFU-S is. BNML and BN cells at these drug concentrations may synthesize DNA, but are no longer able to divide.

In the lower concentration range, however, data on the inhibition of DNA synthesis may be considered to represent the actual drug-induced cell kill. When this assay is used as an indicator for the effects of doxorubicin in vitro, several interesting differences can be observed between the sensitivity of BNML and that of normal bone marrow cells. BNML cells from both spleen and bone marrow exhibit doxorubicin-induced inhibition of DNA synthesis at concentrations of 0.01–1.0 μg · 10^{-7} cells, while normal hematopoietic cells are not sensitive. This implies that in this concentration range doxorubicin is not myelotoxic but exerts significant cytotoxicity on leukemic cells. Higher concentrations lead to killing of both BNML and hematopoietic cells. The observed differences might be attributed to specific properties of BNML cells and normal bone marrow cells with respect to the uptake of doxorubicin into the cell. In both cell types increasing extracellular drug concentrations lead to an increasing uptake of drug, which is, however, more pronounced in BNML cells. The

two cell types do not differ in retention of intracellular doxorubicin when placed in a drug-free incubation medium.

It may be concluded from these data that it is mainly S phase cells that are sensitive to the action of doxorubicin. Pretreatment with ara-C increases the doxorubicin-induced death of BNML and normal bone marrow cells. Still the S phase sensitivity is only valid in the presence of low concentrations of doxorubicin since, according to the LCFU-S and CFU-S data, high concentrations ($10\ \mu g \cdot 10^{-7}$ cells) kill all clonogenic cells and consequently also act on cells in the G_0 and G_2 phases. Therefore, the drug-induced cell kill at high concentrations cannot be accurately determined with the ^{3}H-TdR uptake assay.

When these in vitro observations are translated to the in vivo treatment of this AML, it may be cautiously concluded that the establishment of low tissue concentrations ($0.1-1.0\ \mu g \cdot 10^{-7}$ cells) in bone marrow and spleen will result in doxorubicin-induced kill of leukemic cells without severe harmful effects to normal bone marrow cells. However, in vivo concentrations of doxorubicin in bone marrow of BNML rats, which are obtained following a dose of doxorubicin that is presently used in most AML clinical protocols, are in the range of $1.0-10.0\ \mu g \cdot 10^7$ cells, which produces death of both leukemic and hematopoietic cells [10]. Probably the in vivo bone marrow toxicity of doxorubicin can be safely reduced by reduction of the drug dose. Therefore, the present results may justify an investigation into whether the clinically observed hematological toxicity of doxorubicin can be reduced by the use of lower dosages without any reduction of the antileukemic activity of the drug.

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