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Effects of Daunorubicin and Doxorubicin, Free and Associated with DNA, on Hemopoietic Stem Cells[1]

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ABSTRACT

We have compared daunorubicin (DNR)-DNA with free DNR and doxorubicin (DOX)-DNA with free DOX for their effects *in vivo* in mice on pluripotent stem cells and granulocytic committed stem cells. Dose-survival, time-survival, and recovery curves were obtained after one i.v. injection of either drug.

The dose-survival curves of colony-forming units-spleen (CFU-S) and colony-forming units-committed stem cells (CFU-C) were exponential in shape with both agents. DNR-DNA appeared more toxic to the hemopoietic precursor cells than did free DNR. In contrast, DOX-DNA was less toxic toward CFU-S and as toxic as DOX toward CFU-C. Time-survival curves indicated a minimum level of CFU-S and CFU-C at about 33 hr. After that, the recovery of CFU-S was rapid for DNR-treated mice but remained below 50% of the controls on Day 12 for the DNR-DNA-treated group. In mice previously given injections of DOX or DOX-DNA, the recovery of the CFU-S was more protracted in time with a better recovery in mice treated with DOX-DNA. Both DNR and DNR-DNA induced an initial CFU-C decrease followed by a rapid but transient rise with a maximum on Day 4 after chemotherapy. On Day 12, the CFU-C recovery was still incomplete in both DNR- and DNR-DNA-treated mice. In the groups treated with DOX, the CFU-C recovery was more important after DOX-DNA complex than after free DOX. The results are discussed in view of the "lysosomotropic chemotherapy" hypothesis.

INTRODUCTION

The chemotherapeutic activity of DNR[3] and DOX can be enhanced significantly in L1210 murine leukemia by giving these drugs as complexes with DNA. After i.v. injection of DOX as a DNA complex, the toxicity is significantly reduced in terms of a 50% lethal dose, whereas no such reduction is observed with DNR-DNA (12, 13). This important difference in relative toxicity observed between DNR-DNA and DOX-DNA has tentatively been correlated with a better *in vivo* stability of the DOX-DNA complexes (11).

In an attempt to analyze in more detail the toxicity of DNR-DNA and DOX-DNA and to improve our understanding of the mode of action of the DNA-anthracycline complexes, we have studied quantitatively the cytotoxic effects exhibited by the free and DNA-bound drugs on normal "hemopoietic stem cells" *in vivo*.

The effect of DNR or DOX on the hemopoietic pluripotent stem cells has been analyzed previously (1, 2, 8) after i.p. administration of the drugs.

In this study, we have evaluated the effects on both the pluripotent and committed stem cells of the free and DNA-associated drugs when they are injected i.v.

MATERIALS AND METHODS

In all experiments, 10- to 14-week-old virgin female C57BL/6J mice (19 to 22 g; Centre d'Animaux de Laboratoire, Heverlee, Belgium) were used as the source of bone marrow cells. Sera of OF_1 mice (Animalabo, Brussels, Belgium) or NMRI mice (Centre d'Animaux de Laboratoire, Heverlee, Belgium), injected i.p. with endotoxin, were used as the source of colony-stimulating factor (6).

DOX and DNR were obtained from Rhône-Poulenc, Paris, France. Herring sperm DNA (highly polymerized, type VII Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.15 M NaCl to a concentration of 468 mg/100 ml (w/v). The day before an experiment, an aliquot was autoclaved for 15 min at 120° and left to cool slowly. DNR or DOX were dissolved in 0.15 M NaCl to a final concentration of 40 mg/100 ml (w/v). To prepare the DNA complex, we dissolved 20 mg of drug in 1 ml triple-distilled water and mixed rapidly to 50 ml of DNA (468 mg/100 ml). The drug preparations were further diluted in 0.15 M NaCl when necessary and injected i.v. into recipient mice at intervals varying from 2 to 24 hr or 1 to 12 days before the assays were carried out. Five mice were given injections of each dosage, and 5 mice of the same age were kept uninjected as controls.

Animals were killed by cervical dislocation; the tips of each tibia shaft were cut off, and the marrow plug was flushed out with 1 ml of Hanks' balanced salt solution supplemented with penicillin (20 IU/ml) and streptomycin (20 μg/ml). The bone marrow cells obtained from a pool of 5 tibias were dispersed by gentle pipetting. The nucleated cells were counted in hemocytometers and converted to the number of cells per tibia.

Pluripotent stem cells were assayed *in vivo* according to the method of Till and McCulloch (9). Each cell pool was diluted in Hanks' balanced salt solution to obtain the appropriate cell concentration ranging from 0.8×10^5 to 4×10^5 cells/ml. Seven lethally irradiated mice (850 rads; Cesapan; Barazetti, Monza, Italy) were given injections of 0.5 ml of this cell suspension into the tail vein. Eight days later, the spleens were removed and fixed in Bouin's solution, and the macroscopic colonies were counted. The mean number of colonies per spleen was calculated and the content in CFU-S per tibia was

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[3] The abbreviations used are: DNR, daunorubicin; DOX, doxorubicin; CFU-S, colony-forming units-spleen; CFU-C, colony-forming units-committed stem cells; CFU, colony-forming units; D_{10}, dose of drug required to reduce the colony-forming unit population to 10% of the control value; $D_{1/2}$, dose of drug required to reduce the survival of a colony-forming unit population by 0.5 on the linear part of an exponential curve (14).

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determined from the known number of cells injected into each animal. All these results were normalized with regard to the control values observed in uninjected mice of the same batch.

Granulocytic progenitor cells were assayed *in vitro* in soft-agar culture as described by Quesenberry *et al.* (7) at a bone marrow cell concentration varying between 0.75×10^5 and 3×10^5 cells/Petri dish. In these experiments, sera from mice given injections of endotoxin were used as a source of colony-stimulating factor (6). Five replicate dishes of each set were incubated at 37° for 7 days in a fully himidified atmosphere of 10% CO_2 in air (Forma incubator; Forma Scientific, Inc., Marietta, Ohio). The number of colonies (cell aggregates containing 50 or more cells) were scored using a dissection microscope with indirect lighting (SZ_{III}; Olympus microscope, ×70). The content of CFU-C per tibia was determined from the known number of cells plated and from the mean number of colonies observed per Petri dish. The contents per tibia were normalized to the control values.

Linear regressions were fitted according to the method of least squares to give the general equation

$$\log y = ax + b$$

where x was the dose of drug expressed in mg/kg and y was the fraction of CFU surviving per tibia. D_{10}'s were equal to $-a^{-1} x (1 + b)$ and $D_{1/2}$'s were calculated from the same equation as being equal to $-\log 2 \times a^{-1}$. Slopes were compared by Fisher's 2-tailed F test for analysis of covariance. The differences between the surviving fractions observed after injection of the drugs were analyzed by Student's t test.

RESULTS

Dose-Survival Curves. The survival of pluripotent and committed stem cells was determined by the CFU-S and CFU-C assays 24 hr after i.v. injection of free and DNA-associated anthracyclines as well as DNA alone. The fractions of CFU-S surviving per tibia as a function of drug doses are shown in Chart 1. DNA alone at the doses of 117, 234, and 351 mg/kg (corresponding to the DNA doses administered in mice receiving 10, 20, and 30 mg, respectively, of the DNA complexes per kg) does not appear to influence the pluripotent stem cells.

For the mice treated with DOX and DNR, the dose-survival curves of CFU-S are exponential in shape and are similar. CFU-S are more depressed in mice treated with DNR-DNA than in mice receiving DNR. In contrast, CFU-S are less sensitive to DOX-DNA than to DOX and, moreover, much less sensitive to DOX-DNA than to DNR-DNA.

In Table 1, we have estimated the D_{10} taking into account the possible variations of the initial shoulder as well as these of the slope. The D_{10} of free and DNA-associated DNR for the CFU-S are 12.5 and 8.3 mg/kg, respectively. The D_{10} of DOX are 12.2 mg DOX free per kg and 14.4 mg DOX-DNA per kg. We have also determined the $D_{1/2}$'s which are independent of the initial number of CFU still alive. The $D_{1/2}$ for the CFU-S population are 3.4 and 2.5 mg/kg when DNR was administered as free drug and as DNA complex, respectively ($p < 0.05$). These values are 3.1 mg/kg in mice treated with DOX and 3.6 mg/kg in mice treated with DOX-DNA. The difference observed between DNR-DNA and DOX-DNA is highly significant ($p < 0.01$).

The fractions of CFU-C surviving per tibia 24 hr after we have given different doses are represented in Chart 2. CFU-C values in mice receiving DNA alone are similar to those observed in uninjected mice of the same lot. DOX alone shows a greater effect than DNR toward CFU-C, while DNR-DNA affects the CFU-C population more than does DNR alone. On the other hand, very similar values are obtained after DOX, DOX-DNA, and DNR-DNA.

The D_{10}'s (Table 1) for the CFU-C population are 19.2 mg of DNR per kg or 13.7 mg of DNR-DNA per kg and 12.8 mg of free DOX per kg or 13.2 mg of DOX-DNA per kg, respectively.

The $D_{1/2}$'s of DNR and DNR-DNA populations are 3.9 and 3.1 mg/kg ($p < 0.01$), respectively; DOX and DOX-DNA give equal values (2.5 mg/kg). The difference observed between DOX and DNR is significant at $p < 0.01$, whereas DOX, DOX-DNA, and DNR-DNA do not differ significantly.

Time-Survival Curves. The survival of CFU-S as a function

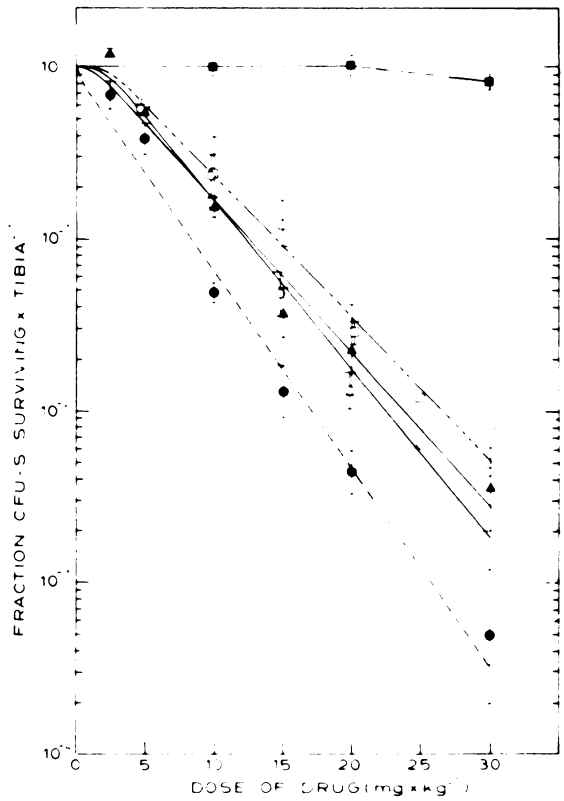

Chart 1. Dose-survival curves for pluripotent stem cells. Groups of 5 mice were given different doses of DNR (▲), DNR-DNA (●), DOX (○), DOX-DNA (□), or DNA (■). Their tibia marrows were assayed 24 hr later for their content of CFU-S in irradiated host mice. The values shown are normalized to the untreated controls. Each *point* represents the geometric mean ± S.E. of 2 to 3 separate experiments from a pool of 5 tibias/experiment.

Table 1

In vivo sensitivity of hemopoietic stem cells to DNR and DOX

D_{10} and $D_{1/2}$ have been derived from Charts 1 and 2.

	CFU-S		CFU-C	
Drug	D_{10}	$D_{1/2}$	D_{10}	$D_{1/2}$
DNR	12.5	3.4	19.2	3.9
DNR-DNA	8.3	2.5	13.7	3.1
DOX	12.2	3.1	12.8	2.5
DOX-DNA	14.4	3.6	13.2	2.5

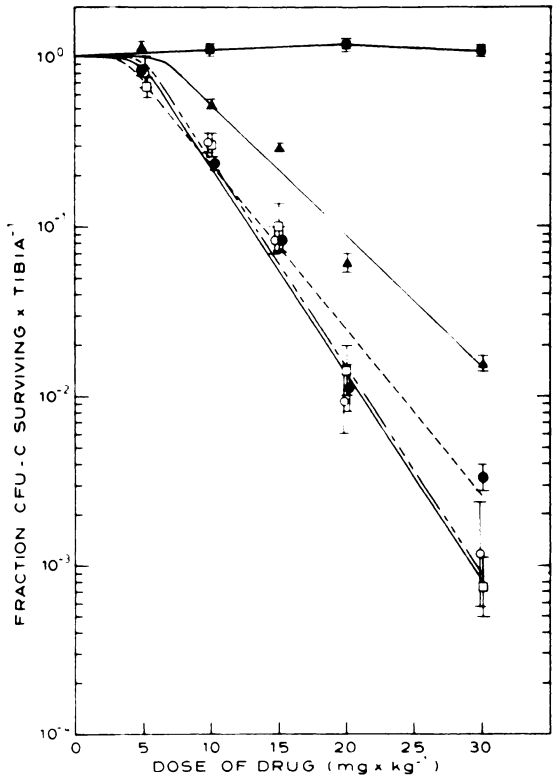

Chart 2. Dose-survival curves for committed myeloid stem cells. Groups of 5 mice were given different doses of DNR (▲), DNR-DNA (●), DOX (○), DOX-DNA (□), or DNA (■). The tibia marrows were assayed 24 hr later for their content of CFU-C. The values shown are normalized to the untreated controls. Each *point* represents the geometric mean ± S.E. of 3 separate experiments from a pool of 5 tibias/experiment.

of time following a treatment by DNR or DNR-DNA at 10 mg/kg and DOX or DOX-DNA at 20 mg/kg are represented in Chart 3.

The decrease of CFU-S after DOX and DOX-DNA is parallel up to 24 hr; at that time, the rate of decrease slows down for DOX-DNA but not for DOX. Up to 24 hr, the CFU-S decrease faster after DNR-DNA than after DNR. At 33 hr, the percentage of CFU-S surviving fall to a nadir of 8 and 5% for DNR and DNR-DNA, respectively, at 10 mg/kg, and 0.4 and 1.8% for DOX and DOX-DNA, respectively, at 20 mg/kg.

Sensitivity and Recovery of Bone Marrow Cells. The marrow cellularity was determined in mice previously treated with DNR (10 mg/kg) or DOX (20 mg/kg), as free and as DNA-associated drugs. The total nucleated cell counts normalized to the control values observed for each experiment are presented in Chart 4.

The survival of the marrow cells in mice given injections of DNR or DNR-DNA is similar for the first 24 hr. After that, the surviving nucleated cells are significantly lower in mice treated with the DNA-associated form. The marrow recovery begins at Day 3 and the difference between the DNR- and the DNR-DNA-treated groups persists to Day 6 ($p < 0.05$ for Days 1 to 6) where the recovery is apparently complete in DNR-treated mice (130% at Day 6) but not in those having received DNR-DNA (80% at Day 6).

The surviving bone marrow cells of mice treated with DOX-DNA are slightly more numerous than those of mice treated with DOX. After a nadir at Day 4, the recovery of the bone marrow cells is better with the DOX-DNA than with the DOX, but the difference is never significant.

Recovery Curves of Hemopoietic Stem Cells. We have studied the recovery of CFU-S in mice treated with either DNR and DNR-DNA (10 mg/kg) or DOX and DOX-DNA (20 mg/kg). Recovery of CFU-S (Chart 5) is biphasic with a rapid phase taking place during the first 3 to 4 days after the nadir followed by a very slow phase or by a plateau.

After DNR, the recovery of CFU-S starting on Day 1 is similar for the free and DNA-associated drug, although the values of CFU-S in DNR-DNA-treated mice remain always lower than those of mice treated with DNR alone. The recovery of CFU-S after DNR-DNA stops on Day 4, and no more progress is observed up to Day 12. After injection of DOX, recovery begins only on Day 2 and is better in mice receiving DOX-DNA. There is a rapid rise up to Day 6 followed by a slower and uncomplete recovery up to Day 12. From Day 6, the partial recovery of CFU-S is similar in the group treated with DNR-DNA (10 mg/kg) and the group receiving DOX-DNA (20 mg/kg).

The recovery of CFU-C population is very rapid after DNR injection (Chart 6) with a pronounced overshoot from Day 2 until Day 4 in animals treated with DNR alone.

The surviving CFU-C fraction drops next on Day 6 to 45% of the control values for both types of DNR followed by a slow recovery. After DOX or DOX-DNA, the CFU-C rise quickly until Day 6 when a plateau is reached for DOX-DNA-treated mice, and a slower increase up to Day 9 characterizes the CFU-C of DOX-treated mice. Recovery of CFU-C is significantly better in

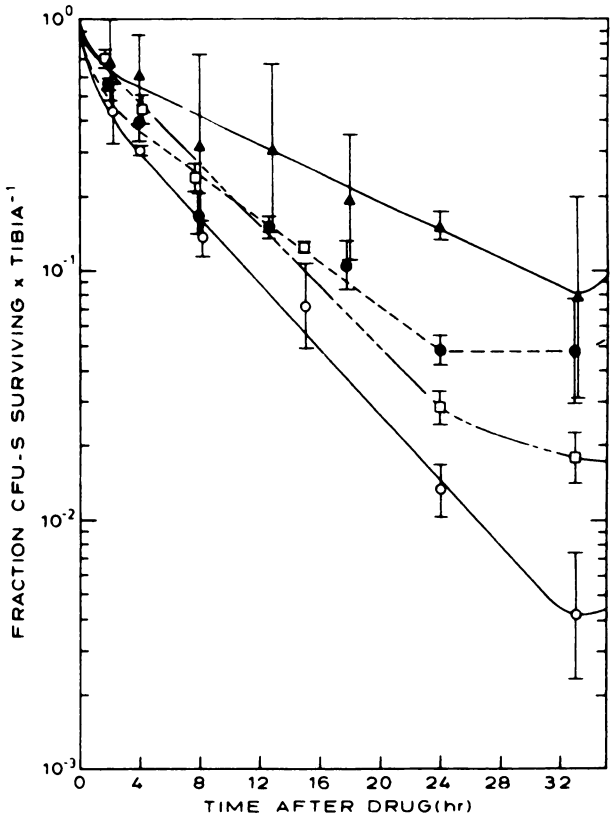

Chart 3. Time-survival curves for pluripotent stem cells. Groups of 5 mice were given either 10 mg DNR per kg (▲), 10 mg DNR-DNA per kg (●), 20 mg DOX per kg (○), or 20 mg DOX-DNA per kg (□) at different times before assay, and the tibia marrows were assayed simultaneously for their content of CFU-S in irradiated host mice. Each *point* represents the geometric mean ± S.E. of normalized values observed in 2 separate experiments.

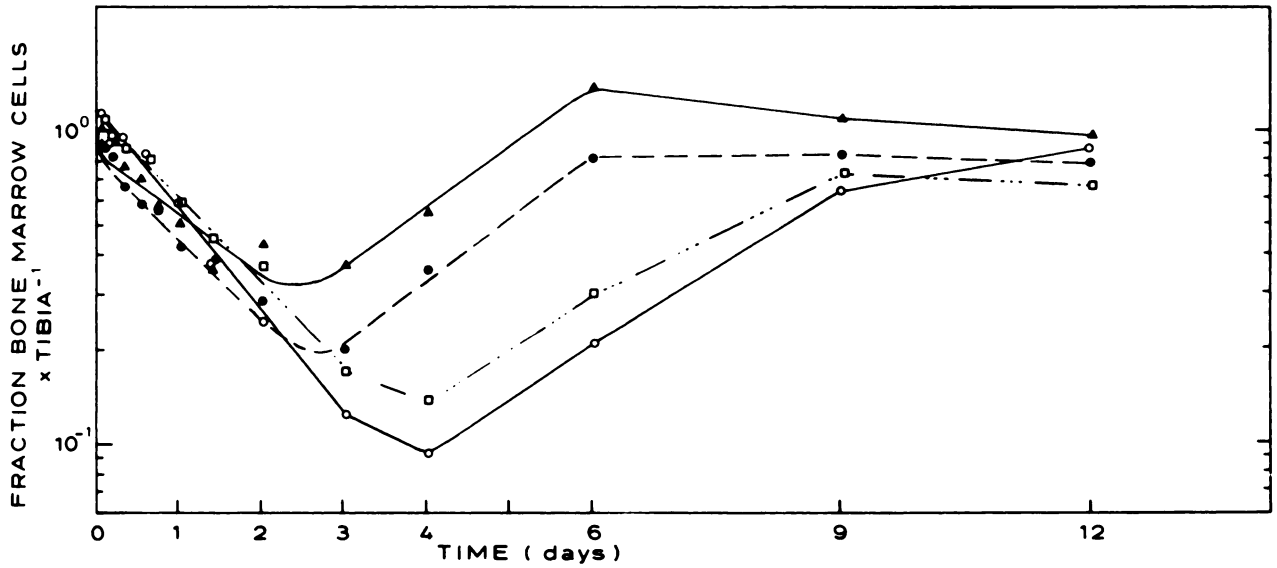

Chart 4. Sensitivity and recovery of bone marrow cells. Groups of 5 mice were given either 10 mg DNR per kg (▲), 10 mg DNR-DNA per kg (●), 20 mg DOX per kg (○), or 20 mg DOX-DNA per kg (□) at different times before assay and their tibia marrows were pooled. The nucleated cell counts from each pool are normalized to the untreated controls. Each *point* represents the geometric mean of 2 or 3 separate experiments.

the DOX-DNA-treated group.

DISCUSSION

The effects on the hemopoietic pluripotent stem cells and granulocytic precursor cells of DNR and DOX given as free drug or as DNA complex have been carefully compared. The activity of DNR in the CFU-S assay expressed as $D_{1/2}$ at 24 hr is about 3.4 mg/kg when the drug is injected i.v. and is very close to that observed by Razek *et al.* (8), who found a value of 4 mg/kg for DNR i.p. DOX seems to be more toxic i.v., since our value of 3.1 mg/kg is significantly lower than those of 9 and 11 mg/kg determined by Razek *et al.* (8), by Blackett *et al.* (2), and by ourselves after an i.p. injection.

The hemopoietic toxicity of DNR-DNA is significantly higher than that of DNR in both assays. The D_{10}'s for the CFU-S are indeed 8.3 mg of DNR-DNA per kg and 12.5 mg of DNR per kg while for the CFU-C they are 13.7 and 19.2 mg/kg, respectively. This higher toxicity of DNR-DNA is noteworthy from the first hr following the drug administration (Chart 3) and remains significant up to 6 days in the CFU-C assay (Chart 6) and up to 12 days in the CFU-S assay (Chart 5). The recovery of the pluripotent stem cells is still incomplete at 12 days after 10 mg of DNR-DNA per kg.

In contrast, DOX-DNA is not more toxic than DOX. The granulocytic committed stem cells are indeed equally sensitive to the 2 forms of the drug as shown by the D_{10} determined at 24 hr: 12.8 mg of free DOX per kg and 13.2 mg of DNA-associated DOX per kg. From Day 1.5 (Chart 6) and up to 4 days, DOX-DNA is less toxic than is DOX at a dosage of 20 mg/kg. DOX-DNA seems to allow a faster recovery of the granulocytic stem cells after an initial equally toxic effect.

DOX-DNA is especially less toxic for the pluripotent stem cells with a D_{10} of 14.4 mg per kg as compared to 12.2 mg of DOX per kg. This decreased toxicity is evident from the first hr (Chart 3) and lasts up to 12 days (Chart 5). DOX and DOX-DNA at 20 mg/kg, like DNR-DNA at 10 mg/kg, induce a permanent effect on the pluripotent stem cells, since this population does not recover its initial level. This toxic effect, less striking with DOX-DNA than with DOX, could be similar to that occurring after busulfan (4).

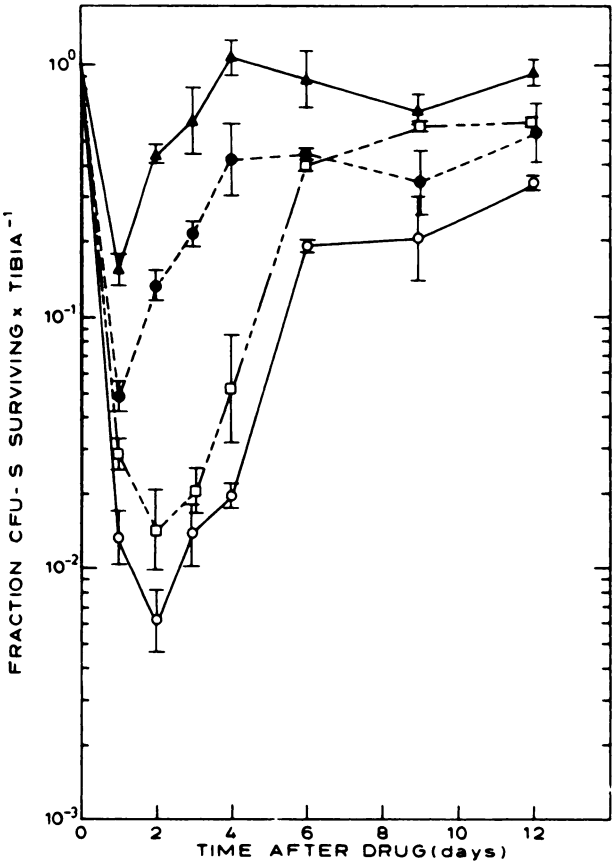

Chart 5. Recovery curves for pluripotent stem cells. Recovery of CFU-S after injection at different days before assay of 10 mg DNR free per kg (▲) or DNR complexed to DNA (●) and 20 mg DOX free per kg (○) or as DNA complex (□). Two separate experiments were carried out for each drug. Each *point* represents the geometric mean ± S.E. of these normalized values from a pool of 5 tibias/experiment.

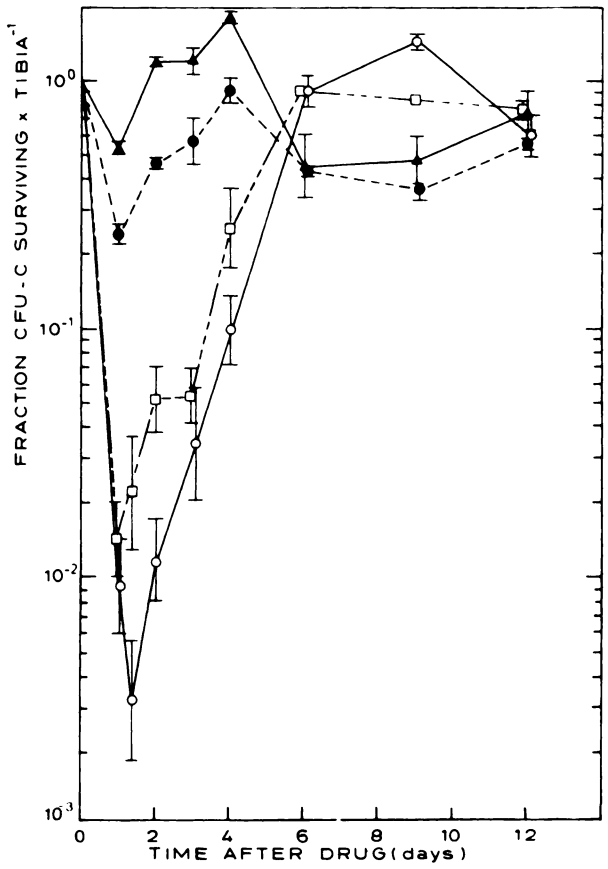

Chart 6. Recovery curves for committed myeloid stem cells. Groups of 5 mice were given either 10 mg DNR per kg (▲), 10 mg DNR-DNA per kg (●), 20 mg DOX per kg (○), or 20 mg DOX-DNA per kg (□) at different days before assay, and their tibia marrows were assayed simultaneously for their content of CFU-C. The values shown are normalized to the untreated controls. Each *point* represents the geometric mean ± S.E. of 3 separate experiments from a pool of 5 tibias/experiment.

The differences observed between DNR-DNA and DOX-DNA were difficult to interpret in view of the very similar *in vitro* affinity of DNR and DOX for DNA which lead us to believe that DNR-DNA and DOX-DNA would be very comparable drug-DNA complexes.

Previously, Ohnuma *et al.* (5) had found that the plasma fluorescence in DNR equivalents 5 min after an i.v. injection of DNR-DNA complex was 60-fold higher than that of an equivalent amount of free DNR. More recent results from our laboratory (3) have shown that the plasma levels of DNR and DOX (free or DNA bound) reached after i.v. injection of DNR-DNA and DOX-DNA are much higher than those obtained after the administration of the free drugs (±40-fold increase for DNR-DNA; ±400-fold increase for DOX-DNA after 10 min in DBA/2 mice). Moreover, a major difference in the stability of the DNR-DNA and DOX-DNA complexes is observed after i.v. injection. DNR-DNA indeed becomes dissociated from its DNA carrier, and one can estimate that ±70% of DNR leaves the blood-stream as a free drug. DOX-DNA seems in contrast to be much more stable, and only about 10 to 15% seems to leave the plasma as free drug.

The hemopoietic toxicity in mice of the DNR-DNA and DOX-DNA complexes can be more easily understood by taking into account these recent data. The higher plasmatic levels of DNR obtained when administering DNR-DNA and the fact that the majority of this DNR leaves the plasma as a free drug may explain the higher toxicity of DNR-DNA for the bone marrow. This increased toxicity could indeed be the result of an increased uptake of DNR by the bone marrow cells without the necessity of releasing DNR from the DNA carrier. This hypothesis is strengthened by the fact that, although the plasma levels of DOX after DOX-DNA are higher than those of DNR after DNR-DNA, the toxicity of DOX-DNA for the hemopoietic stem cells is not increased and is even decreased for the pluripotent stem cells. This may be explained by the stability of DOX-DNA, if we assume that the uptake of DOX-DNA inside the hemopoietic stem cells is decreased when this drug is combined with DNA. According to the lysosomotropic concept of cancer chemotherapy (10), a good drug-carrier complex would be taken up by the cells only through endocytosis, and the drug would be released intracellularly after intralysosomal digestion of DNA. The present results could be explained according to this hypothesis if one assumes that the lower toxicity of DOX-DNA for the pluripotent stem cells is due to their lower endocytic activity for DOX-DNA; this activity should be somewhat higher for the granulocytic committed stem cells. Although this explanation is the most plausible for the effects observed, other possibilities remain to be explored, such as a slow release of DOX from DNA in the extracellular space by a dilution effect. This hypothesis does not, however, allow us to understand the lower toxicity of DOX-DNA for the pluripotent stem cells. The activation of the DNA complex could possibly also occur after digestion of DNA by DNase secreted by the cells or present at their surface. The lower toxicity would in this case be explained by an absence or a very low level of these enzymes in the environment of the hemopoietic stem cells.

Whatever the exact mechanism, our results show clearly that it is possible to modify the toxicity of anticancer drugs by linking them to appropriate macromolecular carriers. Further studies are needed to determine the exact mode of action of DOX-DNA and to prepare better drug carriers.

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