Effect of Dose Fractionation of Daunorubicin on Survival of Leukemic Cells

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ABSTRACT

Mice bearing transplanted AKR leukemia received daunorubicin either as a single dose or as four equally divided doses (0.2 mg/mouse; four times) with the time interval between the divided doses varying from 12 to 36 hr; survival of leukemia colony-forming cells was then assayed. When daunorubicin was administered in any of the fractionated schedules, the dose-survival curve was exponential with a shoulder region demonstrable. As expected, there was significantly less cell killing for the fractionated schedule than for a comparable accumulated single dose; however, with administration of the fourth dose for any interval studied, the increment in cell killing was so large that it was quite similar to that resulting from a single 0.8-mg/mouse dose. We examined the time course of cell killing for the 24-hr fractionation schedule and found greater killing than expected after each subsequent dose with the most pronounced increase occurring after the fourth dose. Possible mechanisms for this effect are discussed.

INTRODUCTION

Many anticancer agents have an exponential dose-survival effect on both malignant and normal cell populations (2). A number of these dose-survival curves are characterized by a shoulder region (9); i.e., at low doses of the agent, increasing doses cause little increase in cell killing compared to that caused by similar increments at larger doses. The importance of the shoulder region on the dose-survival curve for X-radiation has been pointed out repeatedly; for doses within this region, cell repair mechanisms can render a subsequent radiation dose less effective than if the total dose were administered all at once provided sufficient time is allowed between the 2 exposures (11). For radiation, the shoulder region has been attributed to repair processes for sublethal damage. It is probable that similar repair phenomena occur for anticancer agents which damage DNA. However, unlike radiation, chemotherapeutic agents are not deposited within the cell but must pass through the cell membrane and often must undergo conversion to an active species. This pharmacological process probably contributes as well to the shoulder region. Irrespective of the mechanism of shoulder evolution, the prediction of effect remains the same. If low doses are administered in multiple fractions, we would expect a cell population with a significant shoulder region to sustain little if any cell killing at the end of a course of therapy even though the accumulated dose may be quite large.

In this report, we present a quantitative study of the effect of multiple doses of daunorubicin on a transplantable leukemia which we have earlier shown to demonstrate a shoulder region in its dose-survival curves.

MATERIALS AND METHODS

Drugs. We obtained daunorubicin from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. It was dissolved in 0.15 M sodium chloride, and the same diluent was used to prepare the required drug doses that were injected into the tail vein of the mouse in a volume of 0.5 ml.

Mice. AKR mice were obtained from National Animal Laboratories, Creve Coeur, Mo. We used 6- to 8-week-old mice of either sex weighing 18 to 25 g each.

Leukemia Cells. The transplanted AKR line was derived from a mouse with spontaneous AKR lymphoma and transplanted weekly into syngeneic mice as described previously (10). The leukemic mice used in the experiments described below received 10⁶ leukemia cells i.v., prepared from the spleen of a mouse with advanced leukemia, in a volume of 0.5 ml 4 days prior to drug treatment.

Assay for LCFU. The assay was performed on groups of 5 leukemic mice at specified times following drug administration. We removed the femurs of control and treated mice and prepared a monodispersed cell suspension of femoral marrow. After appropriate dilution in a-minimal essential medium (Flow Laboratories, Inc., Rockville, Md.), 0.5 ml/mouse was given via the tail vein to groups of 10 recipients. Eight days later, the spleens of the recipient mice were removed and placed in Bouin’s fixative, and the macroscopic colonies were counted. The number of LCFU from the original donor femur could then be determined (10). We calculated the fractional survival of LCFU by normalizing all results to an untreated control group.

RESULTS

Fractionation of Daunorubicin. We have shown previously that the dose-survival curve of daunorubicin for AKR leukemia cells has an extensive shoulder region (6). The region extends to about 0.2 mg/mouse, which is close to the 10% lethal dose for AKR mice following a single-dose i.v. injection of daunorubicin. We were not concerned with the lethality of the accumulated dose on the specific mouse strain used in these studies but rather with the cellular effect of accumulated doses on a cell line which demonstrated a significant region; in fact, the larger the shoulder region, the better it is for the purpose of this study.

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While 0.2 mg of daunorubicin per mouse are barely cytotoxic, yielding a surviving fraction of about 0.5, a single dose of 0.8 mg/mouse leaves a surviving fraction of between $10^{-4}$ and $10^{-5}$ (6). For this reason, we chose to examine the effects of 4 divided doses of 0.2 mg each. Separate experiments were carried out in which the time interval between the administration of the divided doses was 12, 16, 24, and 36 hr. LCFU were always assayed 24 hr after the last dose. The results of this experiment are shown in Chart 1. The abscissa gives the time of administration of subsequent doses of daunorubicin following the first dose. For example, for the 12-hr interval, the second dose was given 12 hr after the first (LCFU assayed 24 hr thereafter), the third dose was administered 12 hr after the second and 24 hr after the first (LCFU assayed 24 hr after the third dose), and the fourth dose was administered 12 hr after the third or 36 hr after the first (LCFU assayed 24 hr after the fourth dose). The dose-survival curves were exponential when daunorubicin was administered in any of the fractionation schedules, with a shoulder region present in all instances. The extent of the shoulder region increased with increasing intervals. The dashed lines shown in Chart 1 join equivalent accumulated doses and show that, with increasing intervals between administration for accumulated total doses of 0.4 or 0.6 mg/mouse, there was a substantial increase in the surviving fraction of the LCFU. In contrast, the fractional survival for all regimens demonstrates a drastic decrease in survival following the fourth dose (accumulated dose of 0.8 mg/mouse) which was similar to that found following an equivalent single dose $[6 \times 10^{-5}$ (6)].

The results of Chart 1 were unexpected; for example, for the 24-hr interval, we expected nearly complete recovery (repopulation) of the tumor cell population between each fraction. We therefore examined in more detail the kinetics of killing following each of the fractionated doses for the 24-hr interval. Groups of mice received 0.2 mg of daunorubicin per mouse at 0, 24, 48, and 72 hr, and these mice were assayed for LCFU at 6-hr intervals throughout this course of treatment. The results of 2 separate experiments are shown in Chart 2. The first 0.2-mg/mouse dose yielded continuous killing of the LCFU over a period of approximately 12 hr, reaching a minimum of approximately 50% of control. Repopulation of the femoral marrow by LCFU occurred thereafter. However, with each subsequent dose of this drug, killing increased until, after the fourth dose, survival decreased by a striking factor of over 1000. Recovery occurred 12 hr after the first dose; following the second and third doses, recovery did not commence until 18 hr or later; following the fourth dose, no recovery was observed within the 24-hr period, and further cell killing could continue beyond this period. Also shown in Chart 2 is the survival for LCFU following single doses of daunorubicin assayed 24 hr after drug administration and normalized to a 5-day control. In all cases, the survival following an equivalent single dose was lower than that following the fractionated schedule although the 2 survival values of 0.8 mg/mouse are quite similar.

Accumulative Cytotoxicity of Lower Doses. We next determined whether doses of daunorubicin lower than 0.2 mg/mouse also led to an accumulated effect on cytotoxicity. Groups of 4 leukemic mice received i.p. either 0.05, 0.1, 0.15, or 0.2 mg of daunorubicin daily for 4 days. At 24 hr after the last dose, survival of LCFU in the femoral marrow was measured.

In Table 1, we have shown the results and have also presented the 2 possible extremes. In Case A, the survival fraction is that expected when the total 4-day dose is delivered as a single injection; in Case B, the survival fraction is that expected if no accumulation of damage occurs. The latter survival was calculated as the fourth power of the survival fraction found previously (6) for the individual doses.

Data for the 0.05-mg/mouse group are not reported since the mice died of leukemia during the course of treatment. For the 0.1- and 0.15-mg/mouse doses, the fractional survival was slightly less (about a factor of 2) than that expected if no damage had accumulated yet significantly greater than that for an equivalent single dose. By a dose of 0.2 mg/mouse, how-
ever, cell killing had increased appreciably and was nearly 1000-fold greater than if damage accumulation had not occurred.

DISCUSSION

The results reported here indicate that daunorubicin exposure results in the accumulation of nonlethal damage which can be expressed by a later exposure to the agent resulting in increased cell death. This damage can be expressed after an interval of as much as 36 hr. Our studies showed that 0.2 mg of daunorubicin per mouse, which are in the range of the 10% lethal dose for this strain of mouse, resulted in cytotoxic drug levels for up to 12 hr following administration; repopulation occurred thereafter. A second and third equivalent dose given 12 to 36 hr later yielded a small but significant increase in cell killing beyond that expected, i.e., from the time-survival curve following the single dose and the fractionation intervals examined. The fourth dose, however, for all of the intervals studied greatly increased cell killing over that expected for a single 0.2-mg/mouse dose.

A number of possible cellular, biochemical, and pharmacokinetic explanations can be proposed to explain these data. The obvious pharmacokinetic interpretation is that residual drug remains after the first dose and subsequent doses augment the concentration to levels greater than that found following a single 0.2-mg dose alone. This would explain the extended duration of killing found with subsequent doses (Chart 2) as well as the greater extent of killing (6). It has been shown that, while daunorubicin is rapidly removed from the blood following i.v. injection, it is taken up by tissues and only slowly released (12). Recent findings for Adriamycin in humans provide some pharmacological basis for part of this effect (3) in that the plasma disappearance rate for Adriamycin after the sixth dose administered every 8 hr was significantly less than that observed after the first injection. However, in our study, the fourth 0.2-mg dose of daunorubicin elicited a time-survival curve representative of a 0.6- to 0.8-mg dose, and for a 36-hr interval between doses, it seems unreasonable to expect such high levels to have remained. Also, daunorubicin has been shown to have a shorter half-life in cells than the intervals studied here (8). Finally, following the third dose and just before the fourth dose, the residual cell population was increasing (Chart 2); thus, no cytotoxic or even progression-delaying levels of drug could have existed at the time the fourth dose was administered. Therefore, although direct measurement of blood levels of daunorubicin were not made, a pharmacokinetic explanation does not seem plausible.

A possible cellular explanation is that each dose of daunorubicin selects for a cell which is increasingly sensitive to daunorubicin. Such an effect would be unique since continued exposure to an agent usually selects for an increasingly resistant cell type. We are presently examining these cells for increased sensitivity. On a short-term basis, another possible mechanism for increased sensitization is by daunorubicin damage to the cell membrane such that increased drug uptake or, as proposed by Randall Johnson, decreased drug efflux occurs with each additional exposure. We are presently testing these possibilities both in vivo and in vitro.

Finally, there is a biochemical explanation whereby daunorubicin causes nonlethal damage which is ultimately repairable since the divided doses to 0.4 and 0.6 mg/mouse yield significantly less cell killing than that for equivalent single doses. This damage does remain, however, and is susceptible to expression by a subsequent 0.2-mg dose which yields a time-survival curve similar to that expected for a single dose of 0.8 mg/mouse. The difficulty encountered in relating this to the classic examples of sublethal and potentially lethal damage observed following radiation or drug exposure is the long time intervals involved here (1). Both of these documented repair processes are complete in a matter of hours, whereas that noted in our experiments can still be fully expressed even 36 hr later.

The only other authors who have reported a similar phenomenon are Drewinko and Gottlieb (4), who found greater cell killing than expected for divided doses of cis-dichlorodiammineplatinum in vitro; the biochemical or cellular basis was not understood.

These results may be of clinical importance as they indicate that cells can be sensitized following the administration of chemotherapeutic agents such that subsequent administration of further doses of the same (and possibly other) agents can yield an enormous increase in cell killing. An understanding of this sensitization might explain the schedule independence of daunorubicin in which multiple small doses are as cytotoxic as a single large dose (7). This mechanism may underlie why Adriamycin becomes increasingly toxic to cardiac tissue (5).

Finally, the prediction that agents typified by exponential dose-survival curves with shoulder regions are optimally administered as large infrequent doses would be incorrect if multiple small doses could produce the same effect as demonstrated here.

REFERENCES


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