Kinetics of the Lethal Effect of Actinomycin D on Normal and Leukemic Cells

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SUMMARY

Spleen colony assays were used to quantitate the cytotoxic effect of actinomycin D on leukemic cells and normal hematopoietic stem cells. Different doses of actinomycin D were administered to either normal or leukemia-bearing mice, and the surviving fraction of these cell populations was determined. Following single injections of the drug, the killing of both the normal and leukemic cell populations was found to occur over an extended period of time. The kinetics of killing of both cell populations was dose dependent with regard to both the final survival level and the time at which the minimum survival level was reached. The malignant cells exhibited a greater sensitivity than the normal cells. Both this difference in sensitivity and the kinetics of the lethal action of actinomycin D on both cell populations are discussed in terms of the difference in the proliferative states of the two cell populations and the known cellular effects of actinomycin D.

INTRODUCTION

Quantitative information on the degree of killing by a given anticancer agent on both normal and malignant cell populations, as well as the kinetics of this cytotoxicity, is important not only to understand the mechanism of action of the drug but also to define optimal treatment schedules for its use. We recently published a report on the kinetics of cytotoxicity of actinomycin D on a transplantable leukemia (19). Following a single dose of the drug, we found an immediate decrease in leukemic cell survival followed by a prolonged killing of these cells over the subsequent 24- to 36-hr period. We demonstrated that this continued killing of leukemic cells was due to the persistence of cytotoxic levels of actinomycin D in the hosts (19).

In the study reported here, we have extended these findings to the kinetics of killing of both transplanted leukemic cells and normal hematopoietic stem cells using a range of doses of actinomycin D. The results are interpreted in terms of known cellular and biochemical actions of this agent.

MATERIALS AND METHODS

Mice. AKR mice (National Laboratory Animal Company, Crève Coeur, Mo.) of both sexes, 7 to 9 weeks old, and weighing approximately 20 g were used for these studies. They were housed in disposable cages at 5 to 10 mice/cage.

Transplanted Cell Lines. A transplantable syngeneic leukemic (lymphoma) cell line initially isolated from a spontaneous thymic lymphoma 8 years ago at the Ontario Cancer Institute (8) was used in these studies. We obtained this line 4 years ago and have since passaged it weekly from a cell suspension prepared from the spleens of leukemic mice resulting from the passage of the previous week. The spleens were removed, placed on a stainless steel screen (120 mesh), and gently minced with a pair of scissors. Tissue culture medium α-MEM² (Flow Laboratories, Rockville, Md.) was slowly poured over the spleen minced until all material had been passed through the screen. A fraction of this cell suspension was diluted in 0.1 N hydrochloric acid, and the concentration of nucleated cells was determined with the aid of a hemocytometer. Appropriate dilutions were then prepared in α-MEM. The weekly passage was maintained by the i.v. transplantation of between 10⁴ and 10⁵ leukemic cells in a volume of 0.5 ml delivered via the tail vein of recipient AKR mice. The leukemia-bearing mice used in the studies described here received 10⁵ leukemic cells i.v. at the time of the weekly transfer. Four days later, these mice were used for the experiments.

Preparation of Cell Suspensions for Assay. The transplanted leukemia disseminates throughout the recipient mice and proliferates in most of its organs (8). For this study, the femoral marrow was chosen because of the numerically large population of leukemic cells, the results which are readily comparable to data obtained for NCFU (see below), and the relative ease of removal of the femur and its contained cells.

Monodisperse cell suspensions were prepared in the following manner from the femoral marrows of both normal and tumor-bearing mice. Mice were killed by cervical dislocation, their femurs were excised and wiped clean of any adherent tissue, the femoral head was removed, and the distal end was broken at the femoral epiphysis. A 3-ml syringe, attached to a 23-gauge 1-inch needle, was filled from a vial containing 10 ml of α-MEM and partially inserted into the distal end of the femur. The femoral marrow was then flushed into the vial. The syringe was refilled by aspirating the medium back through the femur, the

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²The abbreviations used are: α-MEM, α-minimal essential medium; NCFU, normal hematopoietic colony-forming units; LCFU, leukemic colony-forming units.
femur was removed from the needle, and the needle was inserted into the proximal end of the femur and flushed again. The medium was then drawn through the femur a final time. The femur was removed and discarded and the next unfilled femur was attached to the needle; this was repeated for all femurs within an experimental group using the same vial of medium. Each group consisted of 5 experimental animals (i.e., 1 femur/ml). Appropriate dilutions, determined from preliminary studies, were made with α-MEM.

Assay for LCFU. A 0.5-ml fraction of the appropriately diluted cell suspension of femoral marrow from leukemia-bearing mice was injected i.v. into each of 10 recipient mice. These animals were killed 8 days later, their spleens were removed and placed in Bouin’s fixative, and the number of macroscopic colonies on each spleen was counted. The product of the average number of colonies per spleen and the number of macroscopic colonies per spleen was estimated as LCFU per femur. This assay quantitates those cells that, upon transplantation, seed in the spleen and have sufficient proliferative capacity to produce colonies (10).

Assay for NCFU. The assay for this population of cells has been described previously (17). A 0.5-ml fraction of the appropriately diluted cell suspension of the femoral marrow of normal mice was injected i.v. into each of 15 suprapetally irradiated recipients. Whole-body X-irradiation was delivered in a split-dose schedule of 500 rads initially followed 3 hr later by 400 rads, as suggested by Hellman (13) to minimize destruction of intestinal stem cells, thus increasing the surviving fraction of recipient mice. The recipients were killed 9 to 10 days later, their spleens were removed and placed in Bouin’s fixative, and the number of macroscopic colonies were counted. An estimate of the number of NCFU in the femurs of the donor mice was then calculated as indicated above for LCFU. In irradiated controls not receiving cells, there was an average of 0.2 endogenous colony/spleen.

Actinomycin D. The drug was purchased from Merck, Sharp and Dohme (Rahway, N. J.). Actinomycin D was dissolved in distilled water and diluted in 0.9% NaCl solution; then a volume of 0.5 ml was injected i.v. into the mice. The drug was prepared fresh daily and 2 lots of the drug were used throughout these studies.

RESULTS AND INTERPRETATION

Time-Survival of LCFU. The survival of LCFU as a function of time following the administration of different single doses of actinomycin D was determined. Groups of 5 leukemia-bearing mice were treated with either 2.5, 5, 10, or 20 µg of actinomycin D per mouse. From 30 min to 48 hr later, the femoral marrows of each group were assayed for their content of LCFU. The results are shown in Chart 1. Chart 1A shows the fraction of LCFU surviving per femur for the 1st 8 hr after drug administration. An untreated control group was assayed at 0 time yielding an absolute value of approximately 4 x 10^4 LCFU/femur. The results from all other groups were normalized to this control. For doses up to 10 µg, there appeared to be a decrease in survival of approximately 40% of the LCFU within 30 min of drug administration. This initial reduction to 40% likely results from killing of those LCFU in the DNA-synthetic (S) phase of the cell cycle. Such selective toxicity of actinomycin D for cells in early S has been shown by a number of investigators (1, 7, 11, 12). For the 2.5-µg dose, no further decrease in survival occurred over the ensuing 8 hr; whereas for the 5- and 10-µg doses, a further decrease commenced at 5 and 2 hr, respectively. The plateau region probably results from an inhibition of the progression of LCFU through the cell cycle. It has been shown by a number of investigators that low doses of actinomycin D cause an inhibition of entry of cells into S (1, 15). The doses used here seem in the range of that used for both in vitro and in vivo studies of cell progression inhibition by actinomycin D (2–5, 16). We have previously shown (19) that significant levels of actinomycin D remain in the host for an extended period of time following the administration of dosages in the range of those reported here. It seems probable that the leukemic cells, although blocked in cell progression, continue to incorporate actinomycin D, as has been shown by others (14, 20) and a fraction of these cells, depending upon the dose initially administered, eventually incorporate lethal amounts of the drug. The fact that the duration of the inhibition in cell progression was dose dependent supports this interpretation.

Unlike the lower dose groups, little or no plateau was observed in the survival curve for the 20-µg dose. An exponential time-survival curve was fit to the data for the 20-µg dose using the least-squares method. For this determination, the data obtained for up to 15 hr (Chart 1B) were used. Presumably at high dose levels, actinomycin D is lethal for cells in all stages of the cell cycle. This has been shown previously for L-cells (1).

Chart 1B shows the surviving fraction of LCFU from 8 to 48 hr following these doses. For the lowest dose there was little further decrease in survival of LCFU and, at about 15 hr, a subsequent increase in the surviving fraction of malignant cells in the femoral marrow commenced. For both the 5- and 10-µg doses of actinomycin D, cell survival decreased for approximately 36 hr following which the malignant cell population began to increase in number. With the 20-µg dose, as indicated above, cell survival decreased exponentially throughout the interval of time studied and was below the limit of this assay after 15 hr. For this dose, there was no evidence of recovery for up to 48 hr.

Time-Survival of NCFU. The survival of NCFU as a function of time following the administration of 10, 20, or 40 µg of actinomycin D was next determined. The results are shown in Chart 2. The untreated control contained approximately 2 x 10^5 NCFU/femur. The results from all other groups were normalized to this control. For the 10- and 20-µg doses, there was no apparent immediate killing and cytotoxicity did not become evident until approximately 8 hr after the drug administration. In the case of the lower dose, NCFU survival decreased for approximately 24 hr following which there was an increase in NCFU in the femoral marrow. For the 20-µg dose of actinomycin D, the decrease in NCFU survival continued for
approximately 48 hr, followed by an increase in the surviving fraction of NCFU. We attribute these kinetics to the fact that few NCFU were in the DNA-synthetic phase at the time of administration of the actinomycin D, the majority being in a resting state (6). However, following the destruction of the proliferating stem cell progeny, the stem cells are recruited into cell cycle, and during this recruitment phase sufficient concentrations of actinomycin D were still present in the host to result in the death of these now proliferating stem cells. An alternative explanation is similar to the explanation of the cytotoxic kinetics for LCFU; actinomycin D gradually accumulates in the resting NCFU until a lethal level is reached. Both interpretations are consistent with the dose-dependent minimum found.

For the 40-μg dose, however, there was an immediate decrease in the number of NCFU within 15 min and continued in an exponential manner over a period of 12 hr, after which survival was below the limit of the assay. Thus, NCFU in a nonproliferating state can be killed by high doses of actinomycin D.

DISCUSSION

The current results on the extent and kinetics of killing of LCFU following the administration of actinomycin D corroborate and expand those found previously (19). Following a single dose, continued destruction of the proliferative capacity of both LCFU and NCFU occurs for an extended period of time. These data have been interpreted in terms of the difference between the proliferative states of LCFU and NCFU and is consistent with in vivo and in vitro studies of others.

The postulation that the cellular mode of cytotoxicity is different for high and low doses of actinomycin D explains why this agent was classified as cycle specific by Bruce et al. (9) even though in vitro age-response curves had indicated the drug to be phase specific (1). For low doses, killing is preferentially limited to cells in DNA synthesis and the agent would thus be classified as phase specific; however, the plateau expected in vivo for the dose-survival relationship for phase-specific agents was not found since, as the dose increases, a 2nd mechanism of cytotoxicity becomes evident which results in an exponential dose-survival relationship. This explains the increased sensitivity to actinomycin D found above for proliferating cells when compared to nonproliferating cells; such a differential sensitivity has been shown previously for L1210 cells in vitro (21).

This postulation also has some bearing on the scheduling of chemotherapy. A rational schedule would be to administer a small dose of actinomycin D sufficient to kill proliferating malignant cells in DNA synthesis but ineffective for nonproliferating normal stem cells. In our system,

![Chart 1. Survival of LCFU as a function of time following the administration of different single doses of actinomycin D. A, data for the 1st 8 hr; B, experimental points obtained from 10 to 48 hr. Different symbols represent different experiments. Vertical lines, 1 S.E.](image)
doses would then have to be administered. The time in cumulative toxicity does not occur for the normal stem bearing a large number of malignant cells, subsequent

Since a single dose would not be expected to cure an animal this would correspond to a dose of less than 10 μg/mouse.

With our system, an interval of approximately 48 hr for doses of about 5 μg/mouse would seem appropriate. Studies on mice bearing L1210 leukemia have borne out this prediction (18). We plan to expand these studies and correlate animal survival with cellular survival levels for the NCFU and LCFU cell populations. Also of importance in scheduling is the block sensitivity of normal mouse bone cells. Radiation Res., 14: 213 222, 1961.


REFERENCES

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