Doxycycline in Combination Chemotherapy of a Rat Leukemia

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ABSTRACT

Inhibition of mitochondrial protein synthesis by doxycycline (DC), a tetracycline analogue, has significant antitumor effects in several tumor systems. In the present study, the effects of continuous DC treatment combined with intermittent administration of Adriamycin or 1β-D-arabinofuranosyl cytosine on the growth of a rat leukemia were investigated. The presence of DC retards tumor relapse after 1β-D-arabinofuranosyl cytosine or Adriamycin treatment significantly. DC may therefore be of value in several modalities of antitumor treatment.

INTRODUCTION

The enzymes catalyzing oxidative phosphorylation are composed of several subunits. A number of these subunits are encoded on mtDNA (1) and synthesized by mt-specific translation and transcription processes (2). Inhibition of these processes in proliferating cells halves the cellular concentration of the enzymes concerned during every cell division. In turn, this leads to a lack of oxidative ATP production and, consequently, to proliferation arrest (3). The antiproliferative effects resulting from prolonged inhibition of mt protein synthesis have been demonstrated in several tumor model systems (4–8).

The Roser leukemia of the rat (9) is the most complex in vivo system studied so far. We have shown previously that DC, a specific inhibitor of mt protein synthesis, impairs the growth of this leukemia and ultimately leads to macroscopic eradication of the tumor (10, 11). However, DC-induced tumor eradication leads usually to the death of the rats because of the toxic effects which accompany rapid cell lysis (12, 13), whereas tumor load, which may reduce the mortality from DC-related toxicity, will be arrested sooner by continuous DC treatment. The presence of DC retards tumor relapse after 1β-D-arabinofuranosyl cytosine or Adriamycin treatment significantly. DC may therefore be of value in several modalities of antitumor treatment.

These combinations might result in a lower final tumor load or in a lower rate of tumor relapse and, thus, in a prolonged survival. (a) Unlike Adriamycin or ara-C, DC will also affect noncycling tumor cells. Due to the turnover of the cytoplasm, including mitochondria, the oxidative phosphorylation rate becomes gradually reduced in these cells. If these cells are induced to proliferate after Adriamycin or ara-C treatment, their proliferation will be arrested sooner by continuous DC treatment than that of control cells (14). (b) Adriamycin and ara-C are effective antileukemic agents. The use of these cytostatics will reduce the tumor cell population. The antitumor effect of prolonged DC treatment can thus be achieved at a lower total tumor load, which may reduce the mortality from DC-related tumor lysis.

MATERIALS AND METHODS

Animals and Reagents. Male PVG/c rats (CPB, Nijmegen, The Netherlands) weighing about 250 g were used. DC was a generous gift from Pfizer, Inc. The drug was used in its standard commercial preparation form for i.v. injection (Vibramycin) after dilution to the desired concentration with 0.15 M NaCl. Ara-C was a kind gift from Upjohn, Inc. It was used in its standard preparation form for i.v. injection (Cytarabine). Doxorubicine HCl (Adriamycin) was obtained from Farmitalia and also used in the commercial i.v. preparation form (Adriablastina).

Administration of Drugs. DC was given by continuous i.v. infusion in a dosage which keeps the serum levels constantly between 5 and 10 μg/ml (15). In PVG/c rats, this was achieved by administering 30 and 45 mg of DC/kg/day during the first and second week of treatment, respectively, and giving 60 mg/kg/day during the third and following weeks (10, 11). Control tumor-bearing rats were continuously infused with 0.15 M NaCl only. The continuous infusion device was installed at least 1 wk before tumor inoculation. Continuous infusion lasted maximally 10 wk.

Ara-C or Adriamycin was given once a week by rapid i.v. injection via the continuous infusion device. The treatment schedules were chosen such that about 5 to 10 million nucleated cells were still present per ml of peripheral blood at 2 days after the first administration of these agents at day 18 of tumor growth, i.e., about 15 to 30% of the number of nucleated cells in untreated rats at day 18. To this end, ara-C was given in two portions of 150 mg/kg each, with an interval of 10 h, and Adriamycin as a single dose of 1.5 mg/kg. These relatively low doses (16, 17) were chosen, first, in order to reduce the toxicity of these cytostatics, which allows studies on the effects of DC treatment for lengthy periods, and, additionally, to have a considerable tumor load left on which the effects of DC could be studied within a short period of time. The same considerations led to weekly intervals between the successive ara-C or Adriamycin injections. Control studies showed that the protocol used for treatment with DC, combined with ara-C or Adriamycin, had no severe toxic effects on rats without a tumor.

Tumor System. The T-cell leukemia originally arose as the consequence of irradiation in a Hooded Oxford strain. This strain is syngeneic with the PVC strain used in this study. The pathobiology of the tumor bears much resemblance to human acute leukemia (9). The pattern of tumor spread is complex, but comparable in various series of experiments. After i.v. injection of leukemic cells, tumor growth is first observed in the bone marrow, the peripheral blood, and the spleen. Subsequently, leukemic cells are found in various lymphoid organs while disappearing from the blood in this stage. After inoculation of 10⁶ tumor cells, the rats die about 5 wk later because of the widespread disease which in its terminal stage affects also several nonlymphoid vital organs (10, 11).

Monitoring of Tumor Development. Starting 2 wk after inoculation of 10⁶ tumor cells, all animals were inspected daily. Their weight was recorded, and the general condition was considered. Three times a week, about 50 μl of blood were taken from the tail veins and used to register the number of nucleated cells with the aid of an electronic cell counter. At these time points, the size of the cervical lymph nodes was registered and the general condition was considered. At three time points, the size of the cerebral lymph nodes was estimated by means of palpation. Autopsies were performed on rats which died during the experiments in order to estimate the tumor load.

RESULTS

Effects of Single DC Treatment on Tumor Growth and Host Survival. For reasons of comparison, the results of single DC treatment are shown. The data in Fig. 1 represent the mean of the observations made in the respective groups serving as control.
This explains the prolonged survival of rats treated since Day 14 after tumor inoculation. As illustrated by the data in Figs. 1, B and C, the DC treatment arrested by DC treatment lead to the death of the DC14-rats. The toxic effects, especially on the kidneys, of the rapid disappearance of the tumor once its growth is stopped, the tumor load which is further, tumor growth again follows the same pattern as in untreated controls. Adriamycin thus postpones the fatal effects of the tumor because it temporarily interrupts normal tumor development.

The relation between DC-induced tumor lysis and the time of death of the animals is very strict. As the tumor disappears, the rats lose weight rapidly (about 20 g/day in DC14-rats), and their general condition deteriorates rapidly. At the time of death, the rats have enlarged kidneys, the function of which appears to be seriously affected.

Effects of DC, Combined with Adriamycin, on Tumor Growth and Host Survival. Fig. 2 shows the effects of weekly Adriamycin administration on the survival of control tumor-bearing rats. Per Adriamycin injection, the survival time of these rats is prolonged about 1 wk. Figs. 4 and 5 show that this increased survival time is related to the effects on the tumor load. It takes about 1 wk for the tumor load to return to the the level before Adriamycin was given. If the animals are not treated any further, tumor growth again follows the same pattern as in untreated controls. Adriamycin thus postpones the fatal effects of the tumor because it temporarily interrupts normal tumor development.

The effects of Adriamycin in combination with continuous DC treatment are shown in Figs. 3 to 5. The weekly administration of Adriamycin, combined with DC, clearly leads to a gradually decreasing tumor load in the intervals between successive Adriamycin injections (Figs. 4 and 5), and the rate at which tumor regrowth occurs is lower as DC treatment has lasted longer.

If Adriamycin treatment is stopped, the tumor load which is finally reached in presence of DC is, however, only slightly lower than that found in rats treated with DC only. Like in control rats, Adriamycin treatment delays, therefore, the course of tumor development. It also postpones the final effects of DC on tumor growth and host survival: if DC treatment is continued after the experimental period of 62 days, the animals die because of DC-induced tumor lysis, and if DC treatment is stopped, the tumor develops as in controls.

Effects of DC, Combined with ara-C, on Tumor Growth and Host Survival. Fig. 6 shows the effect of weekly ara-C treatment.

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Fig. 1. Effect of DC treatment on tumor growth and host survival. •, untreated tumor-bearing controls; ○, DC treatment started at Day 0 of tumor inoculation; △, DC treatment started at Day 14 after tumor inoculation. A, effects on survival. Each experimental group consists of 18 rats. B, effects on solid tumor growth. Points, mean of the values found in the rats of A. The size of the cervical lymph nodes is given in arbitrary units based on the mean found in tumor-bearing controls: 0, size at Day 14 after inoculation; 1, at Day 22; 2, at Day 25; 3, at Day 28; 4, at Day 32; 5, at Day 35. C, effects on the nucleated cells in the peripheral blood. The number of nucleated cells is given in millions per ml of blood. Points, mean of the values found in the same rats as in A and B. The SE ranged from 0.2 to 1.9% of the mean values.

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Fig. 2. Effect of Adriamycin treatment on the survival of control tumor-bearing rats. Adriamycin (1.5 mg/kg) was administered at Days 18 and 25 (●); at Days 18, 25, and 32 (▲); or at Days 18, 25, 32, and 39 (▼) after tumor inoculation; •, untreated tumor-carrying control rats. Survival is expressed as the percentage of rats alive in the several experimental groups. ■, n = 12; ▲, n = 9; ▼, n = 12; ○, n = 18.

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Fig. 3. Effect of Adriamycin treatment on the survival of DC-treated tumor-bearing rats. Adriamycin (1.5 mg/kg) was administered at Days 18 and 25 (■, □); at Days 18, 25, and 32 (▲, △); or at Days 18, 25, 32, and 39 (▼, ▶) after tumor inoculation; ◆ and ◼, DC-treated tumor-carrying control rats. Open symbols, rats treated with DC since Day 0 of inoculation; closed symbols, rats treated since Day 14 after inoculation. Survival is expressed as the percentage of rats alive in the several experimental groups. ◆, n = 18; □, n = 9; ▲, n = 8; △, n = 8; ◼, n = 18; ▼, n = 12; ▶, n = 9.

Fig. 4. Effects of Adriamycin treatment on the number of nucleated cells in control and DC-treated tumor-bearing rats. The number of nucleated cells is given in millions per ml of blood. Points, mean of the values found in 8 rats treated with DC since Day 0 of inoculation, in 6 rats treated with DC since Day 14 after inoculation, and in 12 controls. The SE ranged between 0.6 and 1.5% of the mean values. Adriamycin (1.5 mg/kg) was given at Days 18, 25, 32, and 39 after inoculation. ◆, rats not treated with DC; □, rats treated with DC since Day 0 of inoculation; ▲, rats treated with DC since Day 14 after inoculation.

Fig. 5. Effects of Adriamycin treatment on solid tumor growth in control and DC-treated tumor-bearing rats. See the legends to Figs. 1B and 4.

Fig. 6. Effect of ara-C treatment on the survival of tumor-bearing control rats. Ara-C (two doses of 150 mg/kg each, at an interval of 10 h) was administered at Days 18 and 25 (■), or at Days 18, 25, 32, and 39 (▼) after tumor inoculation; ◆, untreated tumor-carrying control rats. Survival is expressed as the percentage of rats alive in the several experimental groups. ◆, n = 18; □, n = 8; ▼, n = 12.

Animals frequently die because of the tumor, and (b) they die because of ara-C-induced tumor cell lysis. The latter accounts, e.g., for the death of 4 of the 5 control rats on Day 39. Ara-C-induced death because of tumor eradication shows a course different from that observed for DC-induced tumor lysis. It is observed at a higher tumor load and occurs within a few hours after ara-C injection. Also in the case of ara-C, the kidneys appear to be severely affected under those conditions.

The effects on survival of DC-treated rats are also significantly smaller in the case of combination with ara-C, as is shown in Fig. 7. However, the death of DC-treated animals is only observed when the rats have become tumor free at the macroscopic level. The moment at which this situation is
reached varies between the several rats of the two DC groups. In view of the strict relation between the time of death and a rapidly declining tumor load, the data on the tumor load of DC-treated rats in Figs. 8 and 9 have been grouped according to the survival of the rats.

The effect of the combination of DC and ara-C appears to be basically the same as that of the combination DC and Adriamycin. DC reduces the tumor load as compared to the controls also between the successive ara-C injections (Figs. 8 and 9). As in the case of Adriamycin, the rate of tumor relapse is lower if DC treatment has lasted longer. However, the absolute rate of tumor reappearance is faster in ara-C-treated DC rats than in Adriamycin-treated DC rats. In turn, this leads not only to a significantly higher tumor load between successive ara-C injections but also to a faster attainment of the maximal tumor load in DC-treated rats after stopping ara-C treatment. This most likely explains the minor effects of ara-C combined with DC on survival as well as the difference in survival between ara-C-treated DC_{p} and DC_{c}-rats. The situation appears to be comparable to that in rats treated with DC only: the antitumor effects of DC lead to tumor lysis-treated death. This effect is more pronounced as the tumor load is larger. Under the experimental conditions of this study, this situation is reached within an experimental period of 62 days, both for treatment with DC only and for the combination of DC with ara-C, but after the experimental period in the case of DC combined with Adriamycin.

**DISCUSSION**

Several aspects of this study indicate that continuous DC treatment may be useful in combined chemotherapy. These concern especially the retardation of the rate of tumor relapse and the reduction of the tumor load between successive treatment with ara-C or Adriamycin.

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**Fig. 7.** Effect of ara-C treatment on the survival of DC-treated tumor-bearing rats. Ara-C (two doses of 150 mg/kg each, at an interval of 10 h) was administered at Days 18 and 25 (■, ○) or at Days 18, 25, 32, and 39 (□, □) after tumor inoculation. Open symbols, rats treated with DC since Day 0 of inoculation; closed symbols, rats treated since Day 14 after inoculation. Survival is expressed as the percentage of rats alive in the several experimental groups. ●, n = 18; ■, n = 9; ○, n = 12; □, n = 18; □, n = 12.

**Fig. 8.** Effect of ara-C treatment on the number of nucleated cells in control and DC-treated tumor-bearing rats. The number of nucleated cells is given in millions per ml of blood. Points, mean of the values found in 12 controls, in 6 rats treated with DC since Day 0 of inoculation and a survival time of 54 to 60 days, in 6 rats treated with DC since Day 0 of inoculation with a survival time of more than 62 days, in 6 rats treated with DC since Day 14 after inoculation with a survival time of 34 to 39 days, and in 6 rats treated with DC since Day 14 after inoculation with a survival time between 46 and 50 days. The SE ranged between 0.5 and 1.8% of the mean value. Ara-C (two doses of 150 mg/kg each, at an interval of 10 h) was administered at Days 18, 25, 32, and 39 after inoculation. ●, rats not treated with DC; ■, rats treated with DC since Day 0 of inoculation; survival time, 54 to 60 days; ○, rats treated with DC since Day 0 of inoculation; survival time, more than 62 days; □, rats treated with DC since Day 14 after inoculation; survival time, between 34 and 39 days; □, rats treated with DC since Day 14 after inoculation; survival time, between 46 and 50 days.

The kinetics of tumor relapse differs between DC-treated rats and the respective control animals, regardless of whether DC is combined with ara-C or with Adriamycin. In controls as well as in DC-treated rats, tumor relapse is, however, faster after ara-C than after Adriamycin treatment. This difference between ara-C and Adriamycin may be based on several mechanisms. For reasons explained in "Materials and Methods," we did, however, not try to find the most effective treatment regimen for either of the two agents.

The difference in relapse rate between DC-treated rats after either ara-C or Adriamycin treatment offers, however, an important clue to explain the data. If tumor cells were involved which belong exclusively to the originally proliferating population, then the rate of proliferation during relapse in the presence of DC should be the same after either ara-C or Adriamycin treatment. Moreover, relapse should be hardly possible when ara-C or Adriamycin is given after a period of DC treatment which is long enough to lead to proliferation arrest (compare Figs. 1, 4, and 8).

During a few days after ara-C or Adriamycin treatment, the growth of the tumor is, however, retarded in the presence of DC. The degree of retardation of tumor growth is clearly related to the duration of pretreatment with DC. The latter suggests...
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that cells are involved in which the oxidative phosphorylation capacity is decreased by DC pretreatment (14). The cells concerned will be arrested relatively fast, whereas the cells which are not effectively affected enough by DC pretreatment are able to proliferate for a longer period.

The results of our study suggest that it may be worthwhile to extend the investigations on the value of DC in combination chemotherapy, e.g., by increasing the frequency, the doses, or the total number of ara-C or Adriamycin injections. Theoretically, this might lead to a considerable and possibly clinically relevant exhaustion of the potentially proliferating compartment. DC treatment combined with conventional anticancer (chemo)therapy has two other possible important advantages as shown in the present study. 

(a) DC treatment reduces the tumor load during the interval between successive ara-C or Adriamycin administrations. This improves not only the quality of life during these periods, but may also increase the effectiveness of treatment with various cytostatics or other modalities. (b) DC treatment may prolong the duration of a remission, since it retards tumor relapse. Indications that mitochondrial agents may be of clinical significance in the latter respect have been described by others before (18).

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