The Effect of Allopurinol on Cyclophosphamide Antitumor Activity

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SUMMARY

We have used the spleen colony assay system and survival duration studies in male DBA/2 mice with P388 leukemia to study the effects of allopurinol pretreatment on the antileukemic activity of cyclophosphamide and its bone marrow toxicity. Allopurinol drinking water (0.5 mg/ml) was given for 7 days prior to cyclophosphamide (10 to 200 mg/kg i.p.). Average daily allopurinol intake per mouse was 1.25 mg (equivalent to 4 mg/kg/day human dosage). Dose-response curves with and without allopurinol pretreatment showed an almost constant 0.9-log increase in the toxicity of cyclophosphamide to leukemia colony-forming units, whereas allopurinol had no effect on the toxicity of cyclophosphamide to normal bone marrow colony-forming units. Parallel survival studies revealed no difference in the antileukemic activity of cyclophosphamide as a result of allopurinol pretreatment. The allopurinol-induced change in the antitumor activity of cyclophosphamide as seen in the spleen colony assay was not explainable on a pharmacokinetic basis. Flow microfluorometric analysis of P388 leukemia tumor cell cycle parameters revealed no change in the blockading effects of cyclophosphamide as a result of allopurinol preexposure. Although we have failed to explain the underlying mechanism of this drug interaction, our data suggest that allopurinol may increase the antitumor activity of cyclophosphamide without increasing its bone marrow toxicity.

INTRODUCTION

Cyclophosphamide is a major anticancer drug with activity against a wide variety of human and animal neoplasms (19). This drug is inactive in vitro, requiring hepatic microsomal enzymes to convert it into its active forms (3, 7, 15). A number of routine drugs (e.g., sedatives, narcotics, antibiotics, etc.) may increase or decrease hepatic microsomal enzyme activity and thereby alter cyclophosphamide metabolism (6, 25). Allopurinol [4-hydroxypyrazolo[3,4d]-pyrimidine], a xanthine oxidase inhibitor which is commonly used for the prophylaxis of hyperuricemia in patients with hematological neoplasms (17, 20), has been said to increase the suppression of granulopoiesis caused by cyclophosphamide and 6-mercaptopurine (2, 5, 18, 24). The Boston Collaborative Drug Surveillance Program has used retrospective data to show that cancer patients, especially those with hematological cancers, treated with a combination of allopurinol and cyclophosphamide experienced greater leukopenia and more thrombocytopenia than those treated with the anticancer drug alone (2, 24). Ragab et al. (22), using in vivo mouse and associated in vitro bone marrow culture techniques, demonstrated that allopurinol at greater than 100 mg/kg caused important depressions of granulocytic colony-forming cells. We have studied the in vivo effects of allopurinol pretreatment on cyclophosphamide antileukemic and bone marrow stem cell toxicity using a mouse spleen colony assay system and tumor-bearing mouse survival studies. In addition we have attempted to relate changes observed in cyclophosphamide antileukemic activity to changes in its pharmacokinetics or its effects on tumor cell cycle kinetics caused by prior treatment of the mice with allopurinol (1).

MATERIALS AND METHODS

Mice. Six- to 8-week old male DBA/2 mice (The Jackson Laboratory, Bar Harbor, Maine) weighing approximately 25 g were used in these experiments.

Mouse Tumor Line. P388 lymphocytic leukemia was supplied by Dr. John Harris (Department of Radiobiology, University of California, San Francisco, Calif.) and serially transplanted as an ascites tumor at weekly intervals (10⁶ cells every 7 days in Medium 199) (Grand Island Biological Co., Grand Island, N. Y.). It was selected for these studies because it more accurately predicts clinical efficacy of anticancer drugs than do other mouse tumor lines (27).

Chemotherapeutic Agents. Allopurinol (Burroughs Wellcome Company, Research Triangle Park, N. C.) in powder form was dissolved in 1.0 N sodium hydroxide, and the pH was adjusted to 10.5 by the addition of 2 N hydrochloric acid. A concentration of 0.5 mg allopurinol per ml solution was obtained by the addition of distilled water. Cyclophosphamide (Mead Johnson and Company, Evansville, Ind.) in powder form was dissolved in sterile water to the desired concentration.

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The Administration Schedule of Chemotherapeutic Agents. The mice were divided into 4 groups of 5 animals. On Day 0, Groups 2 and 4 were started on allopurinol drinking water; Groups 1 and 3 stayed on a regular acid water diet (21). On Day 7, Groups 3 and 4 received cyclophosphamide i.p. (10 to 200 mg/kg) in a constant volume of 0.01 mg/g body weight. All groups were sacrificed on Day 10, and their femurs were assayed for leukemic or normal bone marrow spleen colony-forming units. Mice averaged 2.5 ml/day of either acid or allopurinol drinking water; therefore, daily total allopurinol dosage averaged 1.25 mg/mouse or approximately 50 mg/kg.

LCFU* Assay. Our assay for LCFU was adapted from that of Bruce and van der Gaag (4) and was carried out in the following way: On Day 5, 10⁶ P388 cells were injected into the tail veins of each of the control and allopurinol-pre-treated mice. In the appropriate groups, cyclophosphamide was injected i.p. 2 days (Day 7) after tumor cell injection (when i.p. P388 tumor had a 93+-% growth fraction) (14). On Day 10, the mice were sacrificed, femurs were isolated, and bone marrow cells were washed out with Medium 199. Appropriate dilutions of the femoral bone marrow cells were injected in 0.2-ml volumes into the tail veins of groups of 15 to 20 recipient mice. Nine days later these mice were sacrificed, spleens were removed and fixed in Bouin’s solution, the macroscopic LCFU were counted using a dissecting microscope, and the fractions of surviving LCFU per femur (compared to the controls) were determined.

NCFU Assay. NCFU were assayed in a way similar to that for LCFU except that 3 days after therapy with i.p. cyclophosphamide the mice were sacrificed and appropriate dilutions of normal femoral bone marrow cells were injected i.v. into groups of 15 to 20 whole-body-irradiated (750 rads from a 250-kv source) mice.

Mean Survival Time Studies. Groups of 7 DBA/2 mice were given 10⁶ P388 ascites cells i.v. in a volume of 0.2 ml, and the mean survival times of each of the control and treatment groups were calculated by observing for deaths at 12-hr intervals. The administration schedule of chemotherapeutic agents was similar to that for the LCFU and NCFU assays.

Statistical Analysis. A simple t test was used to determine statistical significance between different experimental groups (8). Dose-response curves were constructed on the basis of linear regression analysis using the Wang 2200 computer.

Determination of Cyclophosphamide Alkylating Metabolites. Allopurinol-pretreated and untreated mice were given cyclophosphamide i.p. in a dosage of 200 mg/kg. At each time point after cyclophosphamide administration, groups of mice were sacrificed by decapitation and their blood was collected in iced, heparinized centrifuge tubes. The blood samples were centrifuged at 2000 rpm for 10 min at 0°, and the resulting 2.5 to 3.5 ml plasma (for each group of 7 mice) were immediately separated and frozen at −20°.

Cyclophosphamide metabolites in the mouse plasma samples were assayed using a modification of the method of Friedman and Boger (11). Two ml of each plasma sample were placed into a centrifuge tube along with 2 ml of 4% perchloric acid. The samples were centrifuged at 200 rpm for 10 min at 0°. One-ml aliquots of the resulting supernatants were transferred to clean test tubes to which were added, in succession, 1 ml of 2 M sodium acetate, pH 5.5, and 0.5 ml of 5% 4-(p-nitrobenzyl)pyridine (Aldrich Chemical Company, Milwaukee, Wis.) in acetone. These mixtures were heated at 100° for 25 min and then cooled in ice for 20 min. To release the colored chromophore for absorbance determinations, each sample was treated as follows: (a) 2 ml of acetone. 5 ml of ethyl acetate, and 2 ml of 1.0 N sodium hydroxide were added; (b) samples were immediately mixed for 20 sec in a Vortex mixer; (c) samples were spun in a tabletop centrifuge for 20 sec; and (d) an aliquot of the upper phase of each sample was placed in a 1-cm quartz cell, and absorbance was read on a Beckman Kintract spectrophotometer at 540 nm 80 sec after addition of the NaOH.

Because cyclophosphamide is inactive in vitro, it was necessary to construct a standard curve using nitrogen mustard in human plasma. Standard curves were determined in quadruplicate. The absorbances obtained from the mouse plasma samples were compared to the absorbances of the standards, and the µg/ml plasma of nitrogen mustard equivalents were determined.

Tumor Cell Cycle Parameter Analysis. Cell cycle parameters for the P388 leukemia cells in ascites form were determined using the FMF at the Biomedical Division of the Lawrence Livermore Laboratories, Livermore, Calif. (under the direction of Dr. J. W. Gray) (13, 26).

The flow facilities used in this study have been described in detail by Van Dilla et al. (26). The monodisperse cell suspension stained with acriflavin, a fluorescent DNA dye, is hydrodynamically focused so that the cells pass in single file through the exciting light beam from an argon ion laser. Each cell upon excitation by the laser beam yields a short fluorescent light pulse the amplitude of which is a measure of the stain content (and therefore DNA content) of the cell. The light pulses are collected by an optical system, converted into electrical pulses, and analyzed by a multichannel pulse-height analyzer. The histogram resulting from the analysis of a large number (10⁶ to 10⁷) of cells gives the distribution of cellular fluorescent stain content over the cell population. Using this technique it is possible rapidly to determine DNA histograms of tumor cell populations following therapy in vivo with anticancer drugs. These DNA histograms were analyzed using computer systems to determine cell cycle parameters for the tumor cells and the exact points of blockade by drugs (i.e., noncancer and anticancer) (13).

The FMF studies for in vivo effects of drugs on cell cycle parameters were performed in the following way: (a) 10⁶ P388 ascites cells were injected i.p. into groups of DBA/2 mice, some of which had been treated with allopurinol in their drinking water at a concentration of one-half the total daily dose per ml (the average DBA/2 mouse drinks 2.5 ml water/day); (b) the anticancer drug was injected i.p. at varying dosages (drug dose required to kill 10 to 50% of a tumor) 2 days after cell administration; (c) sequential cell samples were taken by sacrificing the tumor-bearing mice at frequent time intervals up to 24 hr following drug administration; (d) the ascites cells were harvested and washed.
D. S. Alberts and T. van Daalen Wetters

with media, the RBC were lysed with ammonium chloride buffer, and the counted cells were placed in 10% formalin at a concentration of $5 \times 10^6$ cells/ml; and (e) the cells were stained with acriflavin and FMF analysis was performed.

RESULTS

Allopurinol drinking water pretreatment changed neither the total LCFU or NCFU per femur from those of the untreated control mice; however, allopurinol prior to cyclophosphamide (10 to 27.5 mg/kg) caused a constant increase (approximately 0.7 to 0.9 log) in cyclophosphamide antileukemic stem cell effect. Chart 1 shows the dose-response curve with respect to the fraction of surviving LCFU per mouse femur for cyclophosphamide alone and allopurinol plus cyclophosphamide at dosages of 10 to 27.5 mg/kg.

At cyclophosphamide dosages of 10 to 200 mg/kg, allopurinol pretreatment did not significantly change the dose-response curve of cyclophosphamide for NCFU (Chart 2). The dose-response curves in each case are relatively flat with only little more than a 1-log decrease in NCFU over a dosage range of 10 to 200 mg/kg.

Table 1 shows the mean survival time study results for tumor-bearing mice for allopurinol alone, cyclophosphamide alone, and the combination of these drugs. Those groups of animals treated with allopurinol or acid water and not cyclophosphamide had mean survival times of 5.5 days. At cyclophosphamide dosages of 20, 40, 50, and 70 mg/kg, allopurinol pretreatment had no effect on a change in survival duration over that achieved with cyclophosphamide alone.

As shown in Chart 3, allopurinol prior to cyclophosphamide injection caused no change in the plasma alkylating metabolite curve as compared to that for cyclophosphamide alone. Plasma half-lives for cyclophosphamide alone and allopurinol prior to cyclophosphamide as well as the areas under their respective plasma alkylating metabolite curves were similar (59 µg-hr/ml for cyclophosphamide and 53 µg-hr/ml for the combination).

FMF analysis of P388 leukemia cells following in vivo

<table>
<thead>
<tr>
<th>Cyclophosphamide Dose (mg/kg)</th>
<th>0 mg/kg</th>
<th>20 mg/kg</th>
<th>40 mg/kg</th>
<th>50 mg/kg</th>
<th>70 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide only</td>
<td>5.5 ± 0.00</td>
<td>8.5 ± 0.22</td>
<td>11.6 ± 1.7</td>
<td>14.1 ± 0.55</td>
<td>14.6 ± 0.25</td>
</tr>
<tr>
<td>Allopurinol® plus cyclophosphamide</td>
<td>5.5 ± 0.00</td>
<td>8.1 ± 0.19</td>
<td>11.8 ± 0.28</td>
<td>14.1 ± 0.33</td>
<td>14.5 ± 0.21</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* Allopurinol drinking water (0.5 mg/ml) for 7 days prior to cyclophosphamide.

Diagram 1: Surviving fraction of LCFU per femur as a function of the cyclophosphamide dosage with or without prior allopurinol drinking water treatment. Bars, S.E. Dose-response curves were constructed on the basis of linear regression analysis using the Wang 2200 computer. CFU, colony-forming units.

Diagram 2: Surviving fraction of NCFU per femur as a function of the cyclophosphamide dosage with or without prior allopurinol drinking water treatment. Bars, S.E. Dose-response curves were constructed on the basis of linear regression analysis using the Wang 2200 computer. CFU, colony-forming units.
therapy with allopurinol or allopurinol plus cyclophosphamide revealed no measurable cell cycle blockade by the noncancer drug (Chart 4). There was no evidence of potentiation of the blockading effect of cyclophosphamide. Cyclophosphamide alone caused a late G2 blockade and an early, partial S-phase block, the peak effect occurring at 16 hr after anticancer drug injection (Chart 4).

DISCUSSION

Allopurinol, a xanthine oxidase inhibitor, is the standard drug used for the prophylaxis of hyperuricemia associated with rapid tumor cell lysis secondary to anticancer drug therapy (17, 20). Because 6-mercaptopurine is metabolized to its inactive form (thiouric acid) by xanthine oxidase, it is accepted clinical practice to decrease the dose of 6-mercaptopurine in the presence of allopurinol usage (5, 18). Ragab et al. (22) have used an in vivo-in vitro mouse bone marrow stem cell assay to demonstrate that allopurinol potentiates the suppression of de novo granulopoiesis caused by 6-mercaptopurine. In addition, they showed that allopurinol alone in considerably higher dosage (100 to 400 mg/kg) than would be used in clinical medicine caused severe myelosuppression and inhibition of bone marrow colony formation (22). We have used the in vivo mouse spleen colony assay system to measure the changes in the cytotoxic effects of cyclophosphamide on leukemic and normal bone marrow colony formation as a result of prior exposure of the mice to allopurinol. In this setting allopurinol pretreatment caused an almost 1-log increase in the inhibition of cyclophosphamide of leukemic spleen colony formation at a range of 10- to 27.5-mg/kg dosages. In contrast, allopurinol at dosages equivalent to those commonly used in man for the prophylaxis of hyperuricemia caused no increased toxicity to normal bone marrow stem cell production at a wide range of cyclophosphamide dosage. We conclude that this commonly used mouse model (P388 leukemia in DBA/2 mice) for the screening of potential anticancer activity against human neoplasms (27) does not reflect a decrease in the therapeutic ratio of cyclophosphamide as a result of allopurinol pretreatment.

The mechanisms for the potentiation of the antileukemic effect of cyclophosphamide by allopurinol are at this time still unexplained. Using the standard alkylating agent assay techniques, we were unable to demonstrate a change in the pharmacokinetics of cyclophosphamide in mice as a result of prior allopurinol exposure. Coffey et al. (5), studying the
effect of allopurinol on the pharmacokinetics of 6-mercaptopurine in cancer patients, were also unable to show a change in the anticancer drug half-life or area under the plasma concentration-time curve as a result of the drug interaction effect. Our FMF analysis of the in vivo effects of allopurinol and allopurinol plus cyclophosphamide on P388 leukemia tumor cell cycle parameters demonstrated no allopurinol-associated changes (Chart 4); however, our analytical techniques may not be sensitive enough to detect small changes in DNA repair synthesis caused by the drug interaction. Although allopurinol appears to inhibit purine synthesis (23) and may be converted to an allopurinol ribonucleotide that can inhibit enzymes normally regulated by purine or pyrimidine ribonucleotides (10, 16), there is no evidence that these metabolic effects are related to its apparent interactions with cyclophosphamide. In addition, whereas allopurinol inhibits de novo purine synthesis, it also increases utilization of preformed purines (e.g., hypoxanthine and xanthine) and does not affect purine or pyrimidine nucleotide pool sizes (9).

One possible explanation of the allopurinol-cyclophosphamide interaction effect is that the former drug interferes with the ability of the P388 leukemic cells to repair the sublethal damage caused by cyclophosphamide. The parallel nature of the the cyclophosphamide and allopurinol-cyclophosphamide dose-response curves is a phenomenon often seen in radiobiology as a result of the prevention of DNA repair synthesis. The slopes of such curves reflect the sensitivity of the cells to the drug, and the extrapolated intercept on the abscissa represents the ability with which a cell repairs sublethal damage (12).

It is also possible that the plasma levels of an alkylating agent may not correlate with the antitumor activity of these drugs. It is conceivable that allopurinol alters the fraction of bound RNA, DNA, and protein receptors, thereby potentiating the antitumor effects of cyclophosphamide.

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REFERENCES

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