Antitumor Activity of L-Canavanine against L1210 Murine Leukemia

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ABSTRACT

We have made a preliminary assessment of the antitumor activity of the arginine analog, L-canavanine, in leukemic mice. This analog is known to substitute for arginine in protein biosynthesis in many prokaryotic and eukaryotic systems. Previous studies with cells grown in vitro indicated that canavanine caused a marked inhibition of DNA synthesis and viability. The system used in the present study was C57BL/6 × DBA/2 F1 mice bearing L1210 leukemic cells. Following an i.v. injection of 10 mg canavanine, the t1/2 of canavanine in the serum was estimated at 16 min. This finding suggested that frequent injections of high doses of canavanine would be required for an effect on tumor cell proliferation. DNA synthesis by the L1210 cells, assayed by [3H]thymidine incorporation, fell to 9% of the control value after 12 hourly i.p. injections of canavanine (20 mg each). A constant s.c. infusion of 20 mg/hr for 24 hr caused an 86% inhibition of DNA synthesis. The antitumor activity of canavanine was tested against L1210, using a 24-hr infusion schedule with treatment starting 24 hr after i.p. inoculation of 10^6 cells. An optimal dose of 18 mg/kg body weight produced a median increased lifespan of 44% (p < 0.005). These results suggest that L-canavanine may be useful as an antitumor agent.

INTRODUCTION

L-Canavanine is found in the Leguminosae, a major subfamily of the Leguminosae, and it is the principal free amino acid of numerous legumes (9). It is a guanidinoxy analog of L-arginine, with the formula H2N—C(=NH)—NH——O—CH2—CH2—COOH. The antimetabolic properties of canavanine have been observed in many types of microorganisms, plants, insects, and animal cells (9). Canavanine was found to cause a marked inhibition of RNA and DNA synthesis, with little effect on net protein synthesis. The inhibitory effects were readily reversible or prevented by arginine (9). The basis for toxicity probably stems from the incorporation of canavanine into proteins, as demonstrated in Escherichia coli (11), adenovirus (8), Chlamydomonas reinhardi (7), Walker carcinosarcoma 256 cells (5), and tobacco hornworm larvae (2). The guanidinoxy group of canavanine has a pK of 8.2 as compared to a pK of 10.8 for the guanidino group of arginine (4). This decreased basicity would be expected to have a considerable effect on the activity and structural properties of proteins containing canavanine. This expectation was dramatically confirmed by the appearance of T4 phage possessing giant poby heads as a result of the incorporation of canavanine (1).

A number of amino acid analogs have been shown to possess significant antitumor activity in experimental animals, but few have been active clinically (3). To our knowledge, canavanine has not been tested previously as an antitumor agent, nor has it been shown to inhibit macromolecular synthesis in mammals in vivo. Our preliminary findings with suspension cultures of human lymphocytes suggested that such a study might be rewarding. Phytohemagglutinin-stimulated peripheral blood lymphocytes were readily killed by canavanine, whereas non-dividing human peripheral blood lymphocytes were much more resistant.

The present report demonstrates that L-canavanine can inhibit DNA synthesis in ascitic L1210 in vivo and that this drug significantly prolongs the lifespan of L1210-bearing mice.

MATERIALS AND METHODS

Animals and Cells. Chemotherapy studies were carried out in C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) male mice weighing 20 to 25 g (6 to 10 weeks of age). The parents of these hybrid mice were C57BL/6 and DBA/2. Strain DBA/2 was used as a carrier for the weekly passage of L1210 leukemic cells. The mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. In the chemotherapy studies, mice were inoculated with 10^5 L1210 cells i.p. on Day 0, and death occurred in the untreated animals after a median time of 9 days.

Chemicals. L-Canavanine sulfate was donated by the Calbi-ochem-Behring Corp. (La Jolla, Calif.). The compound was dissolved in phosphate-buffered saline (per liter contains 10 g NaCl, 0.25 g KCl, 1.44 g Na2HPO4, and 0.25 g KH2PO4; final pH of 7.2) and was sterilized by filtration. [3H]Thymidine (15 to 20 Ci/mmol) was purchased from New England Nuclear, Boston, Mass.

Assay of Canavanine in Serum. The blood samples were allowed to clot by storage overnight at 5°. After removal of the clot by low-speed centrifugation, the supernatants were heated for 10 min at 80°. The resultant precipitate was removed by centrifugation (5 min at 10,000 rpm), and the supernatant was assayed for canavanine by the colorimetric method of Rosenthal (10). In a control experiment, canavanine was added to mouse blood and assayed after the above procedure. The recovery was 98%.

Measurement of DNA Synthesis by L1210 Cells. Ascites fluid containing L1210 cells was removed from the peritoneum with a syringe. Aliquots of 2 to 10 µl were diluted with phosphate-buffered saline to 20 µl in microtiter plates, 5 µl of [3H]thymidine (5 µCi) were added, and the mixtures were incubated for 60 min at 37° in a tissue culture incubator flushed with 10% CO2. [3H]DNA was precipitated by the addition of trichloroacetic acid (10% final); the samples were collected and washed with trichloroacetic acid on Whatman GF/C filters and were then counted in a liquid scintillation spectrometer. Assays were carried out in triplicate and corrected for zero-time controls. It was important to assay cellular DNA synthesis...
quickly after the withdrawal of the L1210 cells from the mice. Incorporation of [3H]thymidine decreased rapidly if the cells were stored undiluted at 5° or at 21°.

**Infusion of Canavanine into Mice.** Canavanine (100 mg/ml) was infused at a rate of 0.1 ml/hr through a catheter implanted s.c. over the back. Groups of 5 mice were treated at each dose level together with 6 to 8 control animals and were inspected twice per day for median time of death.

**RESULTS AND DISCUSSION**

Our previous studies with a canine kidney cell line (MDCK) indicated that the effect of canavanine on DNA synthesis and cell viability became maximal when the exogenous concentration ratio of the analog to arginine exceeded 50. Assuming that the serum concentration of arginine in mice is 23 μg/ml (6), we attempted to obtain experimental conditions whereby the serum concentration of canavanine could be maintained for several hr at greater than 1 mg/ml. Under such conditions, an effect of canavanine on L1210 leukemic cells growing in mouse ascites might be expected.

The rate at which free L-canavanine was cleared from the serum of B6D2F1 mice was determined as follows. Ten mg of isotonic, neutralized canavanine (0.1 ml) were injected into the tail vein, and blood samples were obtained at frequent intervals from the retroorbital sinus starting at 2 min after the injection. The concentration of canavanine in the serum was assayed as described in "Materials and Methods." As expected, there was a rapid loss of free canavanine from the serum, with an estimated t1/2 of 16 min (Chart 1).

Based on the above result, it was concluded that frequent injections of canavanine would be necessary in order to attain a sufficient level of the drug to inhibit the replication of L1210 cells growing in vivo. Hourly i.p. injections of 20 mg of canavanine for a period of 6 hr were found to produce only a marginal inhibition of DNA synthesis in the L1210 cells. However, a pronounced inhibition was observed after 12 hourly injections of canavanine. DNA synthesis, assayed in vitro 1 hr after the last dose of canavanine, was inhibited by 91% when compared to cells obtained from untreated control animals.

Canavanine treatment was next extended to 24 hr. To simplify the procedure for administering the drug, the animals were infused with L-canavanine continuously for 24 hr with 20 mg/hr after an initial i.p. injection of 20 mg. L1210 cells were withdrawn from the peritoneum and assayed for DNA synthesis at 12, 24, and 48 hr after the start of infusion. Controls were infused with phosphate-buffered saline. As seen in Chart 2, the inhibition of DNA synthesis in L1210 cells occurred somewhat more slowly than in the previous experiment but nevertheless became quite pronounced by 24 hr after the start of infusion. The average inhibitions were 33% at 12 hr and 86% at 24 hr. Canavanine treatment also caused a pronounced diminution (approximately 70%) in the number of cells found in the ascites fluid at 24 hr. Although most of these cells were viable, this finding suggests that a large fraction of the L1210 cells were killed by canavanine. The reversibility of the canavanine effect on DNA synthesis was evident from the assay at 48 hr, in which only a 10% inhibition of DNA synthesis was observed. At this time, there was no longer any difference in the number of cells (1.5 x 10⁶/ml) in the ascites fluid of control and treated animals.

Canavanine was tested for antitumor activity in mice with early L1210 leukemia. Canavanine was administered as a 24-hr continuous s.c. infusion starting 1 day after inoculation of 10⁶ cells i.p. As shown in Chart 3, an optimal dose of 18 g/kg produced a peak increase in lifespan of 44%. The therapeutic dose range was narrow, and a dose of 24 g/kg caused death due to drug toxicity. We conclude that canavanine does have significant antitumor activity against the L1210 leukemia.

In summary, we have shown that L-canavanine inhibited DNA synthesis by L1210 cells in vivo and significantly increased the

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**Chart 1.** Rate of loss of canavanine from mouse serum after i.v. injection. Canavanine (10 mg in 0.1 ml) was injected into the tail veins of mice. Blood samples were obtained by repeated sampling from the retroorbital sinus. Canavanine was assayed as described in "Materials and Methods."

**Chart 2.** Effect of canavanine on DNA synthesis in L1210 cells. Mice were given injections of 10⁶ L1210 cells; 5.5 days later, 3 animals were infused with canavanine (20 mg/hr for 24 hr after a preinjection i.p. of 20 mg). Two control animals were infused with phosphate-buffered saline. As shown in Chart 3, an optimal dose of 18 g/kg produced a peak increase in lifespan of 44%. The therapeutic dose range was narrow, and a dose of 24 g/kg caused death due to drug toxicity. We conclude that canavanine does have significant antitumor activity against the L1210 leukemia.

In summary, we have shown that L-canavanine inhibited DNA synthesis by L1210 cells in vivo and significantly increased the
lifespan of animals bearing the L1210 leukemia. Preliminary experiments with L1210 and human lymphocytes growing in tissue culture indicate that the inhibition of DNA synthesis by canavanine is reversible by arginine for a period of time (approximately 12 hr), after which this inhibition becomes irreversible. Further studies are needed to determine the mechanism of action, pattern of toxicity, and spectrum of antitumor activity of this interesting arginine analog.

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