Kinetic Analysis of Hepatotoxicity Associated with Antineoplastic Asparaginases

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ABSTRACT

In early studies using high dosages of Escherichia coli asparaginase (10 doses of 1000 IU/kg each) for the treatment of acute lymphoblastic leukemia, hepatotoxicity was a major complication of therapy. Biochemical evidence of hepatic dysfunction occurred in 75% of patients during the first 2 weeks of treatment. Currently, the use of lower dosages of E. coli asparaginase (three doses of 6000 IU/sq m each) has reduced the incidence of hepatic complication. Hepatic dysfunction in the form of severe life-threatening thromboembolic coagulopathy, however, continues to be associated with the use of this enzyme. Our previous work has established that the fatty degenerative changes and hepatocellular dysfunction associated with short-term (1 week) E. coli asparaginase treatment does not occur in mice treated with a glutaminase-free asparaginase from Vibrio succinogenes. In this report, we examined the hepatotoxic effects of prolonged treatment with E. coli and Erwinia carotovora asparaginases and compared the observed toxicities to those observed with the glutaminase-free asparaginase from V. succinogenes. Using a murine model, our data indicate that the hepatotoxicity of E. coli asparaginase parallels the toxicity observed in humans with a rapid increase in liver lipid levels and decreased plasma levels of albumin, antithrombin III, cholesterol, and triglycerides occurring in the first and second weeks followed by a resumption to normal hepatic function during Weeks 3 and 4. In contrast, prolonged treatment of mice with V. succinogenes asparaginase is not associated with significant hepatotoxicity. Er. carotovora asparaginase treatment is associated with an intermediate level of toxicity as indicated by increased hepatic lipid concentration occurring during the second and fourth weeks of treatment. Hepatic function as determined by plasma lipids and proteins was normal in Er. carotovora asparaginase-treated mice.

Our data suggest that the combined physiological depletion of asparagine and glutamine following administration of E. coli or Er. carotovora asparaginases may result in pronounced hepatotoxicity. In contrast, a glutaminase-free asparaginase with potent antilymphoma activity isolated from V. succinogenes is not hepatotoxic even after prolonged treatment. Therefore, it may prove to be a more efficacious antitumor agent in humans.

INTRODUCTION

The discovery that asparaginase from guinea pig serum inhibited the growth of certain asparagine-dependent animal tumors without apparent deleterious effects on normal tissues (4) suggested that this enzyme could be used as a specific and selective antineoplastic agent. Subsequently, a microbial asparaginase from E. coli was shown to act as a potent antileukemic agent. When this enzyme was used in combination with vincristine and prednisone for the treatment of acute lymphocytic or acute undifferentiated human leukemia, an overall remission rate of 93% resulted (27). More recently, the studies of Capizzi et al. (10, 33) have demonstrated a schedule-dependent synergism between high-dose ara-C5 and E. coli asparaginase in the treatment of L5178Y murine leukemia and in the treatment of acute nonlymphocytic leukemia in humans. Another asparaginase isolated from Er. carotovora is presently being used to treat acute leukemia as an experimental drug (21).

While microbial asparaginases have been shown to be effective in the treatment of leukemia, their use is associated with a wide range of host toxicity and pronounced immunosuppression (1, 11, 26, 32, 34). One of the more important clinical hazards associated with asparaginase treatment is hepatic dysfunction (31). Patients treated with E. coli asparaginase have decreased plasma levels of albumin, antithrombin III, cholesterol, phospholipids, and triglycerides. Other indications of asparaginase-induced liver pathology in humans include fatty degenerative changes, delayed bromsulphthalein clearance, and increased levels of serum glutamic-oxaloacetic transaminase and alkaline phosphatase (9).

Some investigators have reported that low dosages of E. coli asparaginase result in limited hepatotoxic complications without compromising therapeutic efficacy (5, 6, 29, 30). Sensitive indicators of hepatic function in some patients receiving low dosages, however, still reveal significant hepatic disease which may result in life-threatening coagulopathy.

The toxic effects of microbial asparaginases may be the result of their capability to hydrolyze both asparagine and glutamine (glutaminase activity) (3, 34). Indeed, patients treated with E. coli asparaginase showed a marked depression of serum levels of both of these amino acids (25). In our previous work, we compared the immunosuppressive and hepatotoxic effects of a glutaminase-free antineoplastic asparaginase from V. succinogenes (12–17) to E. coli asparaginase. The daily i.p. administration of 50 IU of E. coli asparaginase for 3 to 5 days to mice resulted in the pronounced suppression of humoral and cell-mediated immunological responses (16, 17) and pronounced hepatotoxicity (15). The toxicity observed in the murine model closely paralleled the human pathology as evidenced by diffuse microfatty changes within hepatocytes and a marked increase in liver lipid concentration. Liver dysfunction was manifested in decreased plasma levels of albumin, antithrombin III, cholesterol, and triglycerides. V. succinogenes asparaginase treatment was not associated with immunosuppression or significant hepatotoxicity.
The use of asparaginases in combination with other antineoplastic agents (e.g., ara-C and methotrexate) (8, 10, 33) and/or the use of chemically modified asparaginases (19, 22, 35) with extended biological activity may be associated with increased levels of hepatotoxicity. In this context, the studies reported here were performed to analyze the kinetics of asparaginase-induced hepatotoxicity using several antineoplastic asparaginases for prolonged periods of time.

MATERIALS AND METHODS

Animals. The animals used in these studies were 9- to 12-week-old Balb/cGrl mice, originally obtained from the Cancer Research Genetics Laboratory, University of California, and maintained in our laboratory by brother-sister matings.

Asparaginase Preparations. Asparaginase from V. succinogenes was purified to homogeneity as described previously (14). The asparaginase, Ec-2 (Lot 1028A) from E. coli, was obtained from Merck Sharp and Dohme, Point, Pa. E. coli asparaginase was further purified by gel filtration (23) on ultragel Aca 44 (38) (LK Instruments, Inc., Rockville, Md.). Er. carotovora asparaginase (Lot MRE 22) from the Microbiological Research Establishment, Salisbury, England, was provided by Larry Kleinman through the Pharmaceutical Resources Branch of the National Cancer Institute. Asparaginase preparations used in this work were shown to be homogeneous by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate (36).

The relative antilymphoma activity of homogeneous preparations of V. succinogenes, E. coli, and Er. carotovora asparaginases used in these studies was routinely monitored using the 6C3HED lymphosarcoma in C3H mice as reported previously (13).

Enzyme activity was determined by measuring the amount of ammonia produced upon hydrolysis of asparagine as described previously (14) except that 0.01 M sodium phosphate buffer (pH 7.0) was used in the reaction mixture. Enzyme and substrate blanks were included in all assays along with a standard curve prepared with ammonia sulfate. Enzyme activity is expressed as IU (the amount of enzyme catalyzing the formation of 1 µmol ammonia per min under the conditions of the assay). In all experiments, the enzyme activity of V. succinogenes, E. coli, and Er. carotovora asparaginases was determined as the average from triplicate assays of each enzyme preparation. The activity of the 3 enzymes were always determined simultaneously and less than 24 hr before injection. Protein concentrations were determined by the method of Lowry et al. (24). and the specific activities of asparaginase from V. succinogenes, E. coli, and Er. carotovora were determined to be 137, 131, and 292 IU/mg of protein, respectively. The glutaminase activities of these enzymes have been shown to be less than 0.015, 2 to 5, and 9% of the asparaginase activity, respectively (14, 20, 34).

Asparaginase Treatment. Mice between 9 and 12 weeks of age were treated with equivalent dosages of E. coli, V. succinogenes, or Er. carotovora asparaginase. One i.p. injection of 50 IU in 0.20 ml was administered daily for 5 days/week for 1, 2, 3, or 4 weeks. Toxicological studies were performed 18 hr after the last weekly injection. Control animals received injections of 0.01 M phosphate buffer (pH 7.0).

Quantitative Analysis of Total Lipid. Mice were exsanguinated by retroorbital bleeding in order that plasma samples could be obtained. Livers were removed, weighed, and rinsed in 0.85% sodium chloride solution. After being blotted dry with filter paper, livers were cut into small pieces. The tissue was immersed in a minimal volume of chloroform:methanol (2:1, v/v) and homogenized by hand. The resulting extracts were passed through Whatman filter paper to remove remaining tissue fragments. Each extract was then processed according to the procedure of Foch et al. (18) in order to extract and quantitate total lipid.

Determination of Liver-associated Plasma Proteins and Lipids. Antithrombin III activity and albumin, triglyceride, and cholesterol concentra-

trations in plasma were determined using clinical assay reagents and procedures distributed by the Diagnostic Division of Abbott Laboratories. Duplicate standards provided by Abbott Laboratories were run in each assay. Antithrombin III activity is reported as the percentage of control. Albumin concentration is reported in g/dl. Both triglyceride and cholesterol concentrations are reported in mg/dl.

Statistical Analysis. Data were analyzed using Student's t test. The p values used to determine the level of significance of observed differences are reported in the text.

RESULTS

BALB/c mice were treated with daily i.p. injections of 50 IU of asparaginase for 5 days/week for 1, 2, 3, or 4 weeks. E. coli asparaginase treatment resulted in a significant loss in body weight of 19 and 15% during the second and third weeks of treatment, respectively (p < 0.01 and p < 0.001). In the same animals, liver weights were reduced by 31 and 15% during the second and third weeks of treatment (p < 0.001). No significant reduction in animal weight or liver weight was observed during the first week of treatment, and by the fourth week of treatment both weights had returned to normal. No significant reduction in animal weight or liver weight was observed in mice treated with V. succinogenes asparaginase. Er. carotovora asparaginase-treated animals showed a less pronounced reduction in animal weight (6.5% reduction) during the second week of treatment (p < 0.05) and a moderate reduction in liver weight of 15% in the first week (p < 0.01).

A quantitation of total extractable lipid from livers of asparaginase-treated mice (Chart 1) revealed a significant increase in lipid concentration during the first and second weeks of treatment with E. coli asparaginase (p < 0.05 and p < 0.01, respectively). The liver lipid concentrations after 1 and 2 weeks of treatment were 226 and 205% of control animals given injections of phosphate buffer. In contrast, V. succinogenes asparaginase-

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treated mice maintained control levels of extractable lipid throughout the 4-week period (Chart 1). *E. carotovora* asparaginase treatment resulted in a biphasic increase in liver lipid concentration with a statistically significant increase being observed at 2 and at 4 weeks (p < 0.02 and p < 0.01, respectively).

Hepatocellular dysfunction was examined by quantitating several proteins and lipids known to be synthesized by hepatocytes, i.e., albumin, antithrombin III, cholesterol, and triglycerides (Tables 1 and 2). Determination of levels of plasma antithrombin III, a sensitive indicator of hepatocellular function, revealed that only *E. coli* asparaginase treatment caused a significant reduction in this protein. The reduction was pronounced during the first 2 weeks of treatment falling to 74 and 75% of control levels (Table 1). No significant reduction in antithrombin III levels were observed in mice treated with *E. carotovora* or *V. succinogena* asparaginase. Similarly, only *E. coli* asparaginase-treated mice showed significantly decreased levels of plasma albumin (Table 1). These reductions occurred at Weeks 1 and 4 of the scheduled treatment. Decreased concentrations of plasma triglyceride and cholesterol were found to temporarily parallel the increase in total extractable liver lipid and the reduction in antithrombin III in *E. coli* asparaginase-treated mice (Table 2). No significant changes in plasma triglyceride or cholesterol were observed in animals treated with the *E. carotovora* enzyme. *V. succinogenes* asparaginase treatment caused a slight reduction in plasma cholesterol during the first week (22% reduction as compared to control). This was the only parameter of liver function shown to be affected by this glutaminase-free enzyme.

### DISCUSSION

The hepatotoxic effects of *E. coli* asparaginase are well documented in both human and animal systems (7, 9, 26, 31). It has been suggested that the toxic side effects of asparaginas having glutaminase activity may reside in the capability of these enzymes to deplete both asparagine and glutamine. The biosynthesis of asparagine in mammalian systems is primarily mediated through a glutamine-dependent transamidation reaction catalyzed by asparagine synthetase (37). Therefore, glutamine deprivation may block the biosynthetic pathway by which normal cells escape the toxic effects of asparaginase depletion. Studies demonstrating that the mammalian liver is the organ primarily responsible for the homeostatic regulation of asparagine suggest an important physiological role for asparagine synthetase in this organ (37). Asparagine synthetase is an inducible enzyme in rat liver, and its activity increases during regeneration, nutritional deprivation of asparagine or asparaginase therapy (28). The inducible nature of asparagine synthetase may explain why *E. coli* asparaginase treatment is capable of inhibiting only the early wave of mitosis in regenerating rat liver after hepatectomy and not subsequent waves of mitosis (2). Moreover, enzyme induction may be important in alleviating asparaginase-induced hepatotoxicity as suggested by our observations that liver lipid concentrations and plasma levels of lipid and proteins synthesized in the liver return to normal by the third week of treatment.

The present study indicates that treatment of mice for extended periods of time with a glutaminase-free asparaginase from *V. succinogenes* is not hepatotoxic. Our kinetic analysis of *E. coli* asparaginase-induced hepatotoxicity revealed that marked toxicity was evident during the first 2 weeks of treatment followed by a recovery to normalcy. Liver lipid levels increased rapidly during the first 2 weeks correlating with decreased plasma levels of albumin, antithrombin III, cholesterol, and triglyceride (Chart 1; Tables 1 and 2). During the second and third weeks of *E. coli* asparaginase treatment, both animal weight and liver weight were significantly reduced as compared to controls. The most significant absolute increase in total extractable hepatic lipid occurred during the first week of *E. coli* asparaginase treatment followed by a resumption to normal levels. In patients treated with *E. coli* asparaginase for prolonged periods, a similar pattern of recovery is observed (7), suggesting that the murine protein...
model given here is useful for the study of drug-induced hepatotoxicity.

Studies performed by Capizzi et al. (8, 10, 33) have shown a schedule-dependent synergism between E. coli asparaginase and methotrexate or ara-C, suggesting that asparaginase may play an important role in the future development of antineoplastic drug regimens. In particular, the use of high-dose ara-C followed 50 hr later by E. coli asparaginase (600 IU/sq m) has been shown to result in a response in 5 of 7 patients whose disease was refractory to conventional doses of ara-C. The possible combined hepatotoxic effects of E. coli asparaginase and ara-C, however, may limit the therapeutic activity of this drug combination. It is possible that the asparaginase from V. succinogenes, lacking significant hepatotoxic potential, may show this synergistic antileukemic activity without causing increased toxicity.

Lastly, several investigators have attempted to chemically modify various antineoplastic asparaginases in an effort to decrease immunogenicity and extend plasma half-life (19, 22, 35). Extending the duration of asparaginase treatment (20) or the use of modified enzymes may serve to improve therapeutic indices and increase antitumor activity. It may, however, also lead to increased toxicities. Therefore, we are currently chemically modifying the V. succinogenes asparaginase with the hope that the modified enzyme may continue to be less toxic and immunosuppressive and hence be a more effective antileukemic therapy.

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REFERENCES

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