The Toxicity of Escherichia coli L-Asparaginase

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SUMMARY

L-Asparaginase-containing extracts from Escherichia coli have undergone toxicologic evaluation in mice, guinea pigs, rabbits, beagle dogs, and Rhesus monkeys. The preparation elicited acute anaphylaxis in sensitized guinea pigs and produced precipitating antibodies in one of six rabbits. In five of nine batches, temperature elevations of one degree Fahrenheit or more, associated with leukocytosis, were observed in the monkey. With continued treatment the monkey demonstrated varying degrees of weight loss, colitis, leukopenia, and abnormal liver function and histology, which could not be directly correlated with dose or specific activity of the enzyme preparation. The dog was resistant to all toxic effects other than a reversible anemia. Measurement of plasma enzyme concentration after dosing revealed that the monkey maintained significantly higher peak levels than the dog one hour after injection. Both the monkey and dog maintained high plasma activity for the eight hours after the first injection of a twice-daily schedule, and in the monkey the enzyme remained at detectable levels for at least 18 hours.

The role that contaminating bacterial endotoxin may be contributing to the overall toxicity is discussed.

INTRODUCTION

Since the initial observation of the antitumor activity of guinea pig serum by Kidd (23, 24), and Broome's identification of the active principle as L-asparaginase (8—10), much work has been devoted to the isolation of a purified active preparation from Escherichia coli. The therapeutic effectiveness of this bacterially derived enzyme has been confirmed in the 6C3HED lymphoma and the EARAD1 leukemia in mice, primary lymphosarcoma in dogs, and acute lymphoblastic leukemia in children (5, 12, 14, 15, 20, 29, 34, 37, 39). Studies dealing with mechanism of action have shown that certain tumor cells possessing low asparagine synthetase activity are dependent on external sources of L-asparagine and are thus susceptible to the depletion of this amino acid by the action of L-asparaginase (11, 14, 21, 35, 36). Normal cells are thought to be able to synthesize most if not all of their needs and are considered to be independent of external sources. It has been proposed that these quantitative differences in requirement for exogenous L-asparagine will serve as a unique system where therapy can be selectively directed against the sensitive tumor cell while sparing normal tissue.

This communication, which presents the results of toxicologic studies with various batches of L-asparaginase derived from E. coli, demonstrates the particular susceptibility of certain organ systems of the Rhesus monkey to the enzyme preparation. The results are qualitatively compared with toxicity obtained in a Rhesus monkey using a standard preparation of E. coli endotoxin.

MATERIALS AND METHODS

L-Asparaginase (Squibb) was derived from a lysate of Escherichia coli B, formulated in a lyophilized state, 2,000 IU in 10-ml sterile vials. The enzyme was reconstituted in 0.9% saline, 500 IU per ml, prior to use.

In Vitro Assay of L-Asparaginase

Analysis of specific activity was based on the reaction of the enzyme and substrate, L-asparagine, at 37°C. The amount of ammonia liberated was determined spectrophotometrically at 450 mJ by the Nesslerization technic (12, 32). Protein was determined by the method of Warburg and Christian (43). One international unit of L-asparaginase is defined as that amount of enzyme which liberates 1.0 micromole of ammonia per minute at 37°C. The specific activities of the various batches of enzyme tested, TAD 1-9, are presented in Table 1.

Assay for Pyrogenicity

Each batch was assayed for pyrogenicity in male albino rabbits in accordance with the directions in the U. S. Pharmacopeia (42), using an intravenous dose of 200 IU per kg. The enzyme was reconstituted in 0.9% saline, 500 IU per ml, prior to use.

In Vivo Assay of L-Asparaginase

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Assay for Pyrogenicity

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In Vivo Assay of Antilymphoma Activity

Groups of five C3H mice were inoculated subcutaneously with the Gardner lymphosarcoma tumor (6C3HED) ten to twelve days prior to treatment. The mice then received 4 IU per mouse intraperitoneally twice a day for 4 days. The average survival time of untreated animals bearing this tumor was 17 days (range 14—21), while animals treated with L-asparaginase survived for more than 30 days.

1This study was supported in part by Contract PH43-64-932 of the National Cancer Institute, NIH.

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### Table 1

<table>
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<tr>
<th>Batch (TAD)</th>
<th>Dose IU/kg × days on treatment</th>
<th>Number of animals</th>
<th>Specific activity (IU/mg protein)</th>
<th>Total pyrogenicity in rabbits (°C)a</th>
<th>Hematology</th>
<th>Liver</th>
<th>Large intestine</th>
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<tr>
<td></td>
<td></td>
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</table>

Summary of organ toxicity in Rhesus monkeys and Beagle dogs.
aSum of maximum rise in temperature in three rabbits.
bSerum glutamic-oxaloacetic or glutamic-pyruvic transaminases, Sigma-Frankel units.
cAlkaline phosphatase, Bodansky units.

Values of semiquantitation of toxicity

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<th>Toxic range</th>
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<td>&gt; 6 and less than 2 x control</td>
<td>&gt; 2 x control</td>
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<tr>
<td></td>
<td>&gt; 20 and less than 2 x control</td>
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### Assay for Antigenicity

**Capacity to Sensitize Guinea Pigs.** Seven guinea pigs, each weighing 200—300 grams, received 100 IU per kg of TAD-2 intraperitoneally every other day for four doses. Fourteen days after the administration of the first dose, a challenge dose of 100 IU per kg was injected intravenously. Nonimmunized animals were similarly challenged with 100 IU per kg at the same time.

**Capacity to Sensitize and to Produce Antibodies in Rabbits.** After removal of a pretreatment blood sample, six albino rabbits, each weighing 2–3 kg, received 15 IU per kg (TAD-2)
intradermally on Day 1 and intravenously on Days 2, 4, 7, 9, 11, and 14. Blood was again taken on Day 16, and the intradermal injection site was examined for reaction. The serum was tested for precipitating antibodies by agar gel diffusion and in capillary tubes (22). Sera were incubated in dilutions of 1:2 and 1:4 with antigen (TAD-2) in dilutions of 1:5, 1:10, and 1:20 in 0.9% saline at 37°C. The Petri dishes and tubes were examined for precipitate at one hour, and read again after being cooled to 10°C for 24 hours. To test for enzyme inactivating antibodies, 25 IU of L-asparaginase (TAD-2) were added in distilled water to pre- and posttreatment rabbit sera and incubated for one hour at 37°C. The sample was then assayed for residual L-asparaginase activity.

Toxicity Studies

Single-dose toxicity studies were carried out in Swiss albino mice with TAD-1, 2, and 3 using a dose range from 2,000 to 18,500 IU per kg administered intravenously. Hematocrit, red blood count, reticulocyte count, white blood count, and platelet count were followed weekly for a two-week period.

Seventeen Rhesus monkeys (Macaca mulatta), each weighing between 1.94 and 4.0 kilograms, received 350 to 1,000 IU per kg for five days using a twice-a-day dose schedule to simulate the regimen employed in ongoing clinical studies. Four pedigreed beagle dogs (2.42—7.4 kg) were treated with doses ranging from 250 to 2,000 IU per kg per day, administered as two daily intravenous injections. The specific data comparing dose levels, length of treatment, and number of animals are presented in Table 1. The animals were randomly sacrificed after completion of therapy or were allowed a two-week to two-month recovery period during which they were observed for delayed toxicity or reversibility of toxicity. In addition, one monkey received E. coli endotoxin, Bacto Lipopolysaccharide B, 0111:B4, Boivin (Difco), at a dose of 5 mg per kg per day for a total dose of 29 mg per kg. The animals were maintained on a diet of Purina monkey, chow, Gaines dog meal, and water ad libitum. Observations for overt clinical toxicity and body weight were recorded daily. Serial rectal temperatures and white blood counts were recorded hourly after the first dose. Clinical hematology and blood chemistry tests were performed according to the standard Cancer Chemotherapy National Service Center (CCNSC) protocol; serum sodium, potassium, chloride, bicarbonate, and calcium (13) were also determined. All animals underwent autopsy with a gross and histologic examination of all organs and tissues listed in the CCNSC protocol, including the injection site. Liver samples were stained with Oil-Red-O for qualitative determination of fat content (27). For control, two monkeys and one dog were treated with twice-daily saline injections and subjected to the same blood sampling and autopsy protocol.

RESULTS

Antigenicity Studies

On challenge with 100 IU per kg, five of seven sensitized guinea pigs demonstrated acute anaphylaxis, while the nonsensitized control animals showed no toxic symptoms. At necropsy there were no significant findings other than inflation of lungs. Histologic examination of the livers failed to reveal any abnormality.

Using equal parts of 1:5 and 1:10 dilutions of antigen (TAD-2), and a 1:20 dilution of pre- and posttreatment serum, it was found that one of six rabbits developed precipitating antibodies by two weeks. The serum from this and five similarly treated rabbits failed to inactivate the in vitro enzymatic activity of L-asparaginase. Examination of the intradermal injection sites showed them to be without reaction. Histologic examination of the livers revealed a diffuse chronic cholangitis in one of the six animals. There was no evidence of renal cortical necrosis.

One monkey which had received five daily doses of 350 IU per kg (TAD-2) was intravenously challenged with 100 IU per kg (TAD-2) on Day 27 of recovery. There was no evidence of sensitization.

L-Asparaginase Plasma Levels (Charts 1 and 2)

L-Asparaginase activity could not be detected in control dog and monkey plasmas. Following the initial injection of 500 IU per kg, the average maximum enzyme level achieved in five monkeys was 12 IU per milliliter of plasma, with a half-life of approximately eight hours. The second injection of the twice daily schedule produced an accumulative effect, and the enzyme remained at definite detectable plasma level for 18 hours. There was no evidence that the enzyme was either being accumulated from day to day or more rapidly eliminated with continued treatment. One significant finding was that, by giving the total daily dose as a single injection, high plasma enzymatic activity was maintained for a full 24 hours.

In the dog a dose of 500 IU per kg produced a peak average plasma level of approximately 7.5 IU per milliliter one hour postinjection. When compared to the monkey there was a much more gradual removal of the enzyme from the plasma, with about seventy-five percent of the original peak activity recorded at 8 hours.

Chart 1. Plasma L-asparaginase concentration in Rhesus monkeys, followed serially after a dose of 1,000 international units/kg, administered as a single intravenous injection or two divided injections eight hours apart (TAD-4R).

428  CANCER RESEARCH VOL. 29
The Toxicity of Escherichia coli L-Asparaginase

Organ-specific Toxicity (Table 1)

Hematology. The most consistent abnormality found in all monkeys was anemia. Hematocrit and hemoglobin values generally started at 38–42 percent and 12.7–13.8 grams per 100 ml and fell to 28–33% and 7.9–10.6 grams per 100 ml respectively. In the case of TAD-5 the nadir for the hematocrit was 20 percent with a hemoglobin of 5.4 grams per 100 ml. This abnormality could be explained in large part by the serial bleeding for hematologic and chemical determinations, since the two saline-treated monkeys showed reduction of hematocrit to 30 and 33% respectively. One monkey receiving 1,000 IU per kg (TAD-1) demonstrated a delayed onset of anemia, which occurred 10 days after the end of treatment. The onset of reticulocytosis, when seen, was coincident with cessation of dosing and return of hematocrit to baseline values. Reversibility of anemia was observed in all animals allowed a sufficient recovery interval; generally, it required one to three weeks, during which time the animal underwent the serial protocol blood sampling.

Significant leukopenia, white blood counts in the range of 2.5–4.1 X 10^3 cells per cubic millimeter, was noted with those batches of lowest specific activity (TAD-1 and 3) but also occurred transiently with TAD-8 and 9. In the case of TAD-1, the monkey treated with 700 IU per kg per day for five consecutive days, the nadir of leukopenia occurred at Day 36 and failed to return to normal values despite a 68-day posttreatment recovery period. The fall in white count was generally associated with an increase in lymphocyte:granulocyte ratio. Platelet counts fluctuated downward but always stayed above the minimum normal level of 175,000 cells per cubic millimeter. Examination of bone marrow histology at the time of sacrifice failed to reveal any evidence of hypoplasia or maturation arrest in either the red or white blood cell series.

Liver (Fig. 1)

Fatty vacuolization of hepatocytes was observed with seven of the nine batches and showed no direct correlation with either dose level or specific activity. This abnormality was not seen with TAD-2R and 9, the two batches which were nonpyrogenic to the rabbit (Table 1), but these animals underwent two- to four-week recovery periods before sacrifice. Eosinophilic cytoplasmic hyaline deposits resembling “Mallory bodies” (28) were noted in the liver of one animal treated with TAD-i. BSP retention was consistently prolonged in all cases. Abnormal elevations of serum glutamic oxaloacetic and glutamic pyruvic transaminases (SGOT and SGPT) and alkaline phosphatase did occur, but failed to function as a uniform indicator of fatty liver (Table 1). It is significant that the increased BSP retention was readily reversible, and fatty vacuolization was not observed in animals allowed an extended recovery period. BSP values and liver histology were normal in the saline-treated controls.

Gastrointestinal Area

Anorexia and a 3 to 17% weight loss were associated with therapy, and were generally reversible. A fatal hemorrhagic
colitis developed in one monkey receiving 1,000 IU of TAD-1 per kg per day. This degree of colon toxicity was not observed in any other animal, but blood-streaked stools, diarrhea, and erythemic large-intestinal mucosa were noted in other monkeys receiving high specific activity enzyme preparation (Table 1).

**Toxicity of E. coli Endotoxin**

One Rhesus monkey received 5 mg per kg of *E. coli* endotoxin twice a day for a total dose of 29 mg per kg. Immediately after the first injection the animal demonstrated rigor, dyspnea, and restlessness. The white blood count fell from 12,700 to 5,730 cells per cubic millimeter by 30 minutes, with a 1.7°F elevation of rectal temperature by one hour. Pyrexia of two or more degrees Fahrenheit was noted with subsequent dosing. BSP retention rose from less than 1% to 15% by two hours, and BUN was markedly elevated by five hours after the first dose. With continued treatment the animal became anorexic and incurred a 7.3% weight loss by Day 3. There was no evidence of diarrhea or blood-streaked stool. The hematocrit fell to 22% and was accompanied by marked leukopenia of 2,800 cells per cubic millimeter and thrombocytopenia of 77,000 cells per cubic millimeter by the third day of treatment. The BSP retention was 14% at Day 4, and the BUN was 30 mg per 100 ml with a normal creatinine of 0.97 mg per 100 ml. The SGOT and SGPT rose to 110 and 68 Sigma-Frankel units respectively by Day 4, at which time the animal was sacrificed. The significant pathology was localized to the liver with one area of marked replacement with fatty cysts and fat in the portal zones and midzonal intracellular fatty vacuolization.

**Toxicity in Beagle Dogs**

Beagle dogs, three months to one year in age, were tested with TAD-4R and 9 at doses as high as 2,000 IU per kg per day for 14 days. In general these animals tolerated the enzyme preparation better than the monkey at comparable and higher dose levels. They continued to eat and gain weight but developed a mild to moderate anemia, hematocrit of 33% at the nadir, with good recovery. In the one dog receiving a dose of 2,000 IU per kg for 14 days, the anemia was associated with a mild reticulocytosis of 3—4% and as many as 27 circulating nucleated red blood cells per 100 white blood cells. There was no rise in bilirubin and the bone marrow was normal at the time of sacrifice, 17 days after the end of treatment. There was no significant alteration in liver function parameters and no evidence of either hepatic or large intestinal histopathology at the time of sacrifice. The saline-treated dog maintained normal hematology and blood chemistry while undergoing the same serial bleeding protocol.

**DISCUSSION**

The importance of the amino acid L-asparagine to cellular metabolism and protein synthesis is currently undergoing considerable reappraisal. L-Asparagine is grouped with the nonessential amino acids, those that can be synthesized in sufficient quantity by normal cells so as to make exogenous supply unnecessary. In 1956 Neuman and McCoy (30) demonstrated that the Walker 256 carcinosarcoma required L-asparagine for establishment and maintenance of growth in vitro. It has since been shown that the 6C3HED mouse lymphosarcoma, the L5178Y mouse leukemia, and the Jensen sarcoma, have a similar absolute requirement and are susceptible to rapid peripheral depletion of L-asparagine by L-asparaginase in vivo (18, 21, 31). Resistant variants have been characterized as possessing significantly greater L-asparaginase synthetase activity which allows them to grow independently of external sources of the amino acid (11, 21, 35, 36, 41). This work has now extended to the treatment of human cancer, where L-asparaginase has been shown active in the treatment of acute lymphoblastic leukemia (16, 20, 33).

It had been assumed, and indeed the initial toxicologic studies in animals supported the concept, that normal tissues would be resistant to any toxic action of the enzyme (16, 17,
The Toxicity of Escherichia coli L-Asparaginase

FEBRUARY 1969

431
area of 8,000 IU kg per day (J. M. Hill, presented at the Wadley Institute symposium, entitled The Development of L-Asparaginase, April 27, 1968).

The rabbit has been shown to be a second species with remarkable susceptibility. Oetken et al. (33) have demonstrated a syndrome consisting of the sudden onset of convulsions, inability to maintain an upright posture, leading to death. Adamson and Fabro (1) have shown that the E. coli L-asparaginase preparation is teratogenic in the rabbit. Neither of these effects could be related to either pyrogenicity or specific activity of the batch.

Because the enzyme preparation was derived from E. coli, it has been suspected that the toxicity observed may be contributed in part by contaminating bacterial endotoxin. There is as yet no direct proof, but several observations have supported this contention. First there is the significant rise in temperature noted in the rabbits. Fever responses have been noted in the monkey and have in some cases been associated with a biphasic response in peripheral white blood counts consistent with that reported with endotoxin (40). The qualitative toxicities of anemia, leukopenia, BSP retention, and fatty liver, have been seen in both L-asparaginase-treated monkeys and in the one monkey receiving a considerable dose of E. coli endotoxin. However, this is tempered by the failure to find evidence of either a local or generalized Shwartzman reaction in the rabbits.

It is also known that E. coli enzyme preparation does have L-glutaminase activity, which is thought to be characteristic of the particular L- asparaginase (12). The contribution of the hydrolysis of plasma glutamine to the overall toxicity is not known, and this problem will be the subject of future studies.

It is possible that with further purification of the preparation the febrile response and liver toxicity will be eliminated.

ACKNOWLEDGMENTS

The L-asparaginase determinations and agar gel diffusion procedure were performed by Dr. Robert S. Robison and Dr. Bernard Berk of the Squibb Institute for Medical Research. The authors also gratefully acknowledge the technical assistance of Mr. Mansfield Boykin, Miss Annabel Beaty, and Mr. John Rezza, and the efforts of Mrs. Audrey Devendorf in the preparation of the manuscript.

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Fig. 1. Liver histology from a Rhesus monkey five days after completion of treatment with L-asparaginase (TAD-1), 500 international units/kg, twice a day for five consecutive days. Hematoxylin and eosin, × 250.
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