Effect of L-Asparaginase on Mouse Bone Marrow, Assayed by In Vitro Culture

By CLARENCE H. BROWN, III, GEORGE P. CANELLOS AND PAUL P. CARBONE

L-asparaginase administered in therapeutic doses to mice was found to reduce the total number of nucleated marrow cells and colony-forming cells when assayed 4 hours after administration by a methylcellulose bone marrow culture technique. Both marrow cellularity and the fraction of surviving IVCFC/femur were normal by 24 hours after injection, except with very large doses, at a time when there were sufficient quantities of L-asparaginase free or cellularly bound within the marrow to be toxic to the culture system. The enzyme could be removed from the marrow specimens with repeated washings. It is postulated that the bone marrow of the mouse is similar to the regenerating rat liver in that both have the ability to compensate for L-asparagine depletion, even in the presence of active L-asparaginase. The rarity of clinically observed myelosuppression in human subjects could be the result of a similar mechanism.

THE ENZYME, L-ASPARAGINASE has been shown to be a unique agent in the treatment of animal and human neoplasia.1,3 It acts by depleting the nonessential amino acid, L-asparagine from the extracellular environment. The cytotoxic effect was originally considered to be tumor specific. However, clinical trials have demonstrated significant hepatic, renal, pancreatic and central nervous system toxicity in man.3,4 Direct hemopoietic suppression has not been observed in most series.3,5 Pathology studies in animals have also indicated a sparing of the bone marrow from the toxic effects of this drug.1,6

To further examine the effect of L-asparaginase on normal hemopoietic cells in an animal model, the bone marrow from mice was cultured in a methylcellulose medium, 4 and 24 hours following the administration of incremental doses of the agent. Granulocyte and mononuclear cell colonies similar to those reported by Bradley and Metcalf using soft agar7 form in the methylcellulose medium. Marrow cellularity and the number of in vitro colony-forming cells (IVCFC) per femur were reduced with all doses at 4 hours. However, only the highest dose resulted in any suppression at 24 hours. It was also shown that a significant amount of L-asparaginase could be found in the marrow 24 hours after injection which, unless removed with repeated washings of the marrow, was toxic to the in vitro system.
MATERIALS AND METHODS

Crystalline, *Escherichia coli* L-asparaginase (Merck and Co.), reconstituted in 0.85% saline was injected intraperitoneally on separate occasions into two groups of C57Bl/6N male mice in doses of 10, 100, 1000 or 10,000 I.U./Kg.* (hereafter designated as simply I.U.). Saline was injected into control animals. In the first experiment, the mice were sacrificed 4 hours after injection, while in the second experiment, the animals were sacrificed 24 hours postinjection. Both femoral marrows from three animals in each dose group within each time group were pooled in 3 cc. of McCoy's 5A medium. Nucleated cell (n.c.) counts were performed on each marrow pool in duplicate, using separate hemocytometers. The average number of n.c. in the eight hemocytometer chambers was used to determine the n.c./cc. count of each marrow sample. The counts were then converted to n.c./femur. Before the marrow was cultured, each pool was washed six times with McCoy's medium and recounted. One cc. of the washed sample was then diluted with McCoy's medium to a final concentration of 750,000 n.c./cc. The washings were carried out to remove any residual L-asparaginase present in the marrow sample. To test the effectiveness of this procedure a portion of the 24-hour sample from the 1000 I.U. group was left unwashed, counted, diluted and cultured in the same manner as the washed portion of the same sample. In addition, L-asparaginase activity was measured in both portions of this sample and in the washed 4-hour and 24-hour pools from the 10,000 I.U. groups, by the method of Meister.

The in vitro marrow culture method of Worton, McCulloch and Till, with minor variations, was used in these experiments. One-half cc. of the diluted marrow sample to be cultured was mixed in a Falcon plastic test tube with 5.0 cc. of a suspension of methylcellulose, McCoy's medium, fetal calf serum, bovine albumin and colony stimulating factor (L-cell supernatant). Four 1.1 cc. samples, each containing 75,000 n.c. were plated in separate 35 × 10 mm. plastic Petri dishes and incubated in a humidified chamber with 10 per cent CO₂ at 37°C. The plates from the 4-hour experiment were incubated for 10 days and those from the 24-hour experiment for 7 days. The shorter incubation period for the second experiment was chosen for convenience. Both experiments were internally controlled to allow for the difference in incubation. Sufficient numbers of colonies with 50 or more cells are found by 7 days, using this culture system so that accurate counting can be easily done. Data between the two experiments, thus, cannot be compared and was not the purpose of this study. At the end of incubation, the number of colonies containing at least 50 cells (range 50–1000) were counted on each plate with an inverted microscope at 50 times magnification. The colony counts were converted to IVCFC/femur and fraction of control IVCFC/femur surviving.

RESULTS

Table 1 shows the results of n.c. counts of unwashed samples, colony counts produced by washed marrow and the fraction of control IVCFC/femur surviving at 4 hours. There was a slight reduction in marrow cellularity 4 hours after drug administration with all doses, however, colony counts and colony size were comparable to control values for all doses except the 10,000 I.U. in which case a small but significant reduction in colony count, but not size, was observed (Student's t test value 4.63; p < 0.05). All doses used resulted in a reduction in the fraction of control IVCFC/femur surviving at 4 hours.

Table 2 shows the results of the 24-hour experiment. Only the 10,000 I.U. dose produced a decrease in marrow cellularity. The number of colonies and colony size for all doses were comparable to controls. The uniform reduction

*Approximately equivalent to 0.2, 2, 20 or 200 I.U./mouse.
**Table 1.—Marrow Cellularity, Colonies per Plate and Fraction of Control in Vitro Colony-forming Cells (IVCFC) per Femur Surviving at 4 Hours**

<table>
<thead>
<tr>
<th>Dose (I.U./Kg.)</th>
<th>0</th>
<th>10</th>
<th>100</th>
<th>1000</th>
<th>10,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marrow n.c. × 10⁷ per femur †</td>
<td>2.35</td>
<td>1.60</td>
<td>2.10</td>
<td>1.50</td>
<td>1.90</td>
</tr>
<tr>
<td>Colonies per plate ‡</td>
<td>99.3 ± 2.7</td>
<td>101.5 ± 3.1</td>
<td>96.5 ± 2.1</td>
<td>93.3 ± 3.7</td>
<td>76.3 ± 3.2</td>
</tr>
<tr>
<td>Fraction of control IVCFC per femur surviving 1,§</td>
<td>1.0</td>
<td>0.69 ± 0.02</td>
<td>0.86 ± 0.06</td>
<td>0.59 ± 0.02</td>
<td>0.62 ± 0.06</td>
</tr>
</tbody>
</table>

* Following L-asparaginase administration.
† Average of eight hemocytometer chamber counts, multiplied by dilution factor; converted to n.c./femur.
‡ Mean ± SE.
§ Based on mean IVCFC/femur for control animals.
∥ Student’s t test.

in colony counts for the 24-hour experiment was a result of the shorter incubation period. At 24 hours only the highest dose produced any reduction in the fraction of IVCFC/femur surviving, however, even this was not statistically significant.

The results of the washing experiment are shown in Fig. 1. The washed portion of the 24-hour marrow specimen from the mice receiving 1000 I.U. produced an average of 74.5 colonies per plate, whereas the unwashed sample produced an average of only 36.3 colonies, the difference presumably due to removal of residual L-asparaginase by washing. A further control study revealed that the washing of normal, untreated mouse marrow did not significantly alter the number of colonies formed per constant inoculum of cells. In addition to a reduction in the number of colonies formed from unwashed marrow, there was also observed a diminution in the size of colonies with no greater than 200 cells per colony being seen. L-asparaginase activity could not be detected in the washed sample of any of the measured samples; however, a small quantity of activity was present in the unwashed portion of the 1000 I.U. group 24 hours after drug administration.

**Discussion**

These experiments indicate that therapeutic doses of L-asparaginase in mice result in a decrease in total bone marrow cellularity, which is reflected in both decreased n.c. counts, as well as a reduction in the fraction of control IVCFC/femur surviving. However, this effect is seen early and within 24 hours is no longer apparent, except with very large doses. Our data suggest that by 24 hours after a single injection of L-asparaginase there is adaptation to L-asparagine depletion by normal mouse bone marrow cells exposed to L-asparaginase. This phenomenon of adaptation was suggested by Becker and Broome who showed that liver regeneration could occur in hepatectomized rats receiving L-asparaginase, suggesting that repletion of intracellular L-asparagine had occurred even in the presence of L-asparacinase. That this
Table 2.—Marrow Cellularity, Colonies per Plate and Fraction of Control in Vitro
Colony-forming Cells (IVCFC) per Femur Surviving at 24 Hours *

<table>
<thead>
<tr>
<th>Dose (I.U./Kg.)</th>
<th>0</th>
<th>10</th>
<th>100</th>
<th>1000</th>
<th>10,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marrow n.c. × 10^7 per femur †</td>
<td>2.20</td>
<td>2.20</td>
<td>2.00</td>
<td>2.25</td>
<td>1.60</td>
</tr>
<tr>
<td>Colonies per plate †</td>
<td>77.8 ± 3.6</td>
<td>73.0 ± 1.8</td>
<td>77.8 ± 2.7</td>
<td>74.5 ± 4.1</td>
<td>82.5 ± 4.3</td>
</tr>
<tr>
<td>Fraction of control IVCFC per femur surviving †,‡</td>
<td>1.0</td>
<td>0.93 ± 0.02</td>
<td>0.91 ± 0.07</td>
<td>0.98 ± 0.05</td>
<td>0.77 ± 0.10</td>
</tr>
</tbody>
</table>

* Following L-asparaginase administration.
† Average of eight hemocytometer chamber counts, multiplied by dilution factor; converted to n.e./femur.
‡ Mean ± SE.
§ Based on mean IVCFC/femur for control animals.
|| Student’s t test.

is the case is suggested by the demonstration of an increase in the L-asparagine synthetase of normal rat liver during L-asparaginase treatment. The apparent sparing of the bone marrow to the toxic effects of the enzyme previously observed in animals and humans very likely represents a similar biochemical mechanism.

The observation that the culturability of mouse marrow is reduced by L-asparaginase has been reported previously. However, the doses used were very large and in some instances even greater than the doses which are therapeutic in murine tumor systems. In addition, the removal of residual L-asparaginase from explanted marrow was not attempted, which may have yielded suboptimal results.

That fewer and smaller colonies are formed in plates into which unwashed

Fig. 1.—Effect of L-asparaginase on mouse bone marrow assayed by in vitro culture. Number of colonies per 75,000 nucleated marrow cells, comparing unwashed bone marrow with marrow washed six times. Each point represents a single plate.
L-ASPARAGINASE

marrow is placed indicates that L-asparaginase is present either in plasma or bound to cell surfaces for at least 24 hours after a single dose in the mouse. L-asparagine is a necessary ingredient in the culture system used, if maximal growth is to be obtained.9,13 In each plate there is approximately 20 \( \mu \)g of L-asparagine, an amount that would be rapidly hydrolyzed by a very small quantity of L-asparaginase contaminating the system. In addition, glutaminase activity which accompanies the L-asparaginase would be capable of destroying glutamine, another amino acid essential to many tissue culture systems. It is thus evident that studies of the biologic effect of L-asparaginase assayed by cell culture techniques should include the precaution of adequately removing contaminating enzyme.

ACKNOWLEDGMENTS

We are grateful to Miss Eleanor Stashick and Mrs. Marla Cohen for technical assistance.

REFERENCES

Effect of L-Asparaginase on Mouse Bone Marrow, Assayed by In Vitro Culture

CLARENCE H. BROWN III, GEORGE P. CANELLOS and PAUL P. CARBONE