L-Asparagine Requirement and the Effect of L-Asparaginase on the Normal and Leukemic Human Bone Marrow

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SUMMARY

The bone marrow of patients with acute lymphocytic leukemia, acute myelogenous leukemia, and metastatic cancer (normal marrow) was evaluated for L-asparagine dependence with the use of the in vitro test of Sobin and Kidd. All of the marrows including the normal ones showed L-asparagine dependence. There was a direct correlation between the concentration of L-asparaginase and depression of cell function as measured by the in vitro test. It was demonstrated that the enzyme itself and not contaminating materials was responsible for this effect. The above results are consistent with the fact that L-asparaginase treatment produces an initial antileukemic effect in the majority of patients with acute leukemia. However, the in vitro test was not useful in predicting remission.

Asparagine synthetase activity was determined in normal and acute leukemic marrow. The activity prior to L-asparaginase treatment was invariably low and in approximately one-half of the bone marrows no activity could be demonstrated. Again, there was no correlation between asparagine synthetase activity and the production of remission with L-asparaginase.

The major limitation of L-asparaginase treatment in acute leukemia is the rapid development of clinical resistance. In preliminary studies of serial determination of the in vitro test and asparagine synthetase activity of the bone marrow during L-asparaginase treatment, a decreasing L-asparagine requirement and increasing asparagine synthetase activity were observed. The decreasing L-asparagine requirement presumably results from an increase in asparagine synthetase activity. This would explain the rapid development of resistance in clinical leukemia and also the lack of functional impairment of the normal bone marrow.

The significance of these findings as well as other possible mechanisms of clinical resistance to L-asparaginase are discussed.

INTRODUCTION

The amino acid L-asparagine is considered to be nones-

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sential for normal mammalian tissues. It is, however, a requirement for certain neoplastic tissues as demonstrated for mammalian cells in culture (5, 9, 10) and for certain lymphoma cells in rodents (8). Subsequently, it has been amply confirmed that L-asparaginase, which hydrolyzes L-asparagine, exerts antitumor effects on a number of lymphomas in rodents (1, 8) and dogs (6, 13) and in ALL2 in man (6, 11). Sobin and Kidd (15) developed an in vitro test for L-asparaginase requirement of ascites tumor cells. This test involves the measurement of the viability of cells as determined by radioactive precursor incorporation into macromolecules, as affected by culture media wherein the amount of L-asparagine is varied. By this test, L-asparagine requirement of transplanted tumors as measured in vitro has been closely correlated with the in vivo antitumor effects of L-asparaginase (11, 15). The following is a report of the application of this test and related studies of L-asparaginase metabolism to clinical material.

MATERIALS AND METHODS

Adenosine triphosphate, L-glutamine, L-aspartic acid, and L-asparagine were purchased from Sigma Chemical Co., St. Louis, Mo. L-Aspartic acid-4-14C (specific activity, 10 mCi/mmmole) and L-asparagine-4-14C (specific activity, 10 mCi/mmmole) were obtained from Calbiochem, Los Angeles, Calif. Fetal calf serum, streptomycin, penicillin, and L-glutamine (for cultures) were purchased from Hyland Laboratories, Inc., Los Angeles, Calif. Uridine-5-3H (specific activity, 20 Ci/mmmole) was obtained from Schwarz Bio-Research, Orangeburg, N. Y. L-Asparaginase of Escherichia coli was supplied by E. R. Squibb and Sons, New Brunswick, N. J.; Truett, Dallas, Texas; and Merck and Co., Inc., Rahway, N. J.

The Test of Normal and Leukemic Bone Marrow Cells for L-Asparagine Requirement in Vitro ("In Vitro Test")

This was based on the observations of Sobin and Kidd (15), with mouse leukemia, and it has been applied to human cells by Oettgen et al. (11). The procedure was adapted and modified as follows.

1 The abbreviations used are: ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia.
Cell suspensions were prepared from heparinized normal and leukemic bone marrow by mashing through a screen (25 mesh) with a glass rod and rinsing with fetal calf serum. The cell suspensions were then sedimented with dextran (pyrogen-free; molecular weight, 200,000 to 300,000; final concentration, 0.4%). After sedimentation for 30 min the tube was returned to an upright position for 5 min. White cell-rich supernatant was withdrawn and adjusted to a concentration of 0.5 X 10^6 cells/ml in Eagle's minimum essential medium containing 30% fetal calf serum, 1.3 mM L-glutamine, 70 units each streptomycin and penicillin, and 2 μCi uridine-5-3H. Two ml each of this culture preparation were pipetted to culture tubes, and the cultures were divided into groups of 4 or 5 tubes each depending upon the availability of cells. For routine assays the 1st group contained 0.5 mM L-asparagine, the 2nd group 0.08 i.u. E. coli L-asparaginase/ml, the 3rd group 1.5 i.u., the 4th group 15 i.u., and nothing was added to the 5th group which served as a control. These tubes were incubated in an incubator with 5% CO_2 in air at 37°. At various time intervals, the cultures were interrupted by washing the cells twice with 10 volumes of 0.9% NaCl solution each time, and the incorporation of uridine into nucleic acids was measured in a liquid scintillation spectrometer after precipitation of the samples with 0.5% sodium dodecyl sulfate and 5% cold trichloroacetic acid on a Millipore filter with 0.45 μm pore size.

In some studies, dialyzed serum containing no free L-asparaginase and uridine was used. Although this increased the amount of uridine incorporation, it did not change the relative values in the in vitro test. The effect of various concentrations of L-asparagine ranging from 0.2 to 20 mM was tested. The uridine uptake of the samples with 0.2 mM L-asparagine was similar to those of 2 mM, but with levels equal to or over 5 mM there was decreasing uridine uptake. Thus, the concentration of L-asparagine, 0.5 mM, was chosen for our routine assays.

Cultures preincubated with L-asparaginase for 3 hr before adding uridine showed no difference in uridine incorporation from those in which uridine was added to cultures at the beginning of incubation.

Thirteen patients with AML and 9 patients with ALL were included in the studies. All patients with ALL and 4 of 9 patients with AML had received known effective antileukemic treatment before L-asparaginase treatment was introduced. Eleven patients with metastatic carcinoma were treated with L-asparaginase. In all of these patients, the in vitro test was performed on the aspirated bone marrow prior to treatment and, in some patients, it was performed at intervals during and following treatment.

Asparagine Synthetase of Human Tissues

Preparation of Enzyme. Fresh human tissues obtained surgically were rinsed with 0.9% NaCl solution and homogenized in 1 volume of 0.02 M Tris buffer, pH 7.4. Human bone marrow was obtained by aspiration. Red blood cells were removed by hypotonic shock for 30 sec (4) and washed 3 times with 100 volumes of 0.9% NaCl solution.

The packed cells were then extracted sonically with 1 volume 0.02 M Tris buffer. The homogenates were centrifuged at 100,000 X g for 20 min and the supernatant was removed and used as enzyme preparation.

L-Asparaginase Synthetase Assay. The incubation mixture for measurement of formation of L-asparagine contained L-aspartic acid-4,14C, 0.2 μmole (0.1 μCi/μmole); adenosine triphosphate, 2 μmoles; L-glutamine, 4 μmoles; MgCl_2, 2 μmoles; Tris buffer, 10 μmoles at pH 8.0; and an appropriate amount of enzyme in a total volume of 0.2 ml. The reaction was started by the addition of L-aspartic acid. After the mixture was incubated at 37° for 60 min, the reaction was stopped by the addition of cold perchloric acid to a final concentration of approximately 4%. The protein was precipitated in an ice bath for 15 min and removed by a brief centrifugation. The product, L-asparagine, was separated from the supernatant by paper electrophoresis at pH 8.6 in a barbitone-acetate buffer. The radioactive spots were located and identified with authentic compounds by a Packard radiochromatogram scanner. The areas containing radioactivity were then cut and counted directly for 14C in a Packard liquid scintillation spectrometer.

RESULTS

The results of the in vitro test in selected typical examples of acute leukemia and normal bone marrow are shown in Chart 1. For both the acute leukemic cells and normal bone marrow cells, there was considerably greater uridine uptake in the samples to which L-asparagine was added than in those in which it was omitted. The addition of L-asparaginase to the media further depressed uridine uptake and, in general, the greater the concentration of L-asparaginase the greater the depression of uridine uptake.

The variability of uridine uptake among patients with normal marrows, ALL, and AML as a function of time is presented in Chart 2. These are the values from the samples to which 0.5 mM L-asparagine was added. Note that the overall values were comparable for the normal bone marrow and for the ALL cells whereas the uptake was approximately 2-fold greater in AML cells. For the normal bone marrow, the uptake was approximately linear at 20 and 40 hr of incubation whereas for the leukemic cells there was a decreasing uptake after 20 hr. Determinations prior to 20 hr indicated linear uptake during the first 20 hr. By morphological and trypan blue exclusion studies, there was evidence of a variable amount of cell death at 40 hr in the acute leukemic cells. Accordingly, subsequent determinations were made at 20 hr of incubation unless otherwise indicated.

Percentage of inhibition by L-asparaginase or L-asparagine dependence was calculated as follows:

\[
\text{% inhibition by L-asparaginase} = \frac{\text{Uridine uptake with L-asparagine} - \text{Uridine uptake with L-asparaginase}}{\text{Uridine uptake with L-asparagine}} \times 100
\]

The percentage of inhibition by the addition of varying concentrations of L-asparaginase in patients with ALL or...
AML and in the normal bone marrow as related to the concentration of L-asparaginase in the media is presented in Chart 3. In patients with ALL, low concentrations of L-asparaginase (0.08 i.u./ml) produced little or no inhibition in the majority of patients, but at higher concentrations (1.5 i.u./ml) almost all of the patients had significant inhibition (median, 42%). The results in patients with AML were generally similar at the low concentration, but the inhibition curve was steeper and at 1.5 i.u./ml a median inhibition of 62% was obtained. The normal bone marrow cells were similarly inhibited by different concentrations of L-asparaginase, with median values of 23% inhibition at 0.08 i.u./ml and 54% at 1.5 i.u./ml.

Since the L-asparaginase used to date is not completely free of impurities, the inhibitory effect on normal cells in vitro raised the question that the effect was not due to the enzyme but to contaminating material such as endotoxin. Various techniques were used to evaluate this possibility.

L-Asparaginase was relatively thermostable and boiling for 10 min did not completely inactivate the enzyme; in numerous experiments, 15 to 30% of the enzyme activity was retained. There was a good correlation between the reduction in L-asparaginase activity as measured by enzyme assay and the decrease in inhibition of uridine uptake as measured by the in vitro test (Chart 4).

In addition, the effect of perchloric acid denaturation of L-asparaginase on the in vitro test of normal bone marrow was studied (Table 1). The enzyme was denatured by adding perchloric acid (final concentration, 4%) and precipitated in an ice bath for 30 min. The neutralized perchloric acid (after removing potassium perchlorate) had no adverse effect on uridine uptake, and perchloric acid completely eliminated the inhibition produced by L-asparaginase. Enzyme assay also showed complete elimination of enzyme activity. The addition of L-asparagine to this sample enhanced uridine uptake, whereas the inhibitory effect of undenatured L-asparaginase could not be reversed by adding L-asparagine (Table 1). In fact, a 10-fold increase of the routine concen-
Effect of L-Asparaginase on Human Marrow

possibility still remains that the procedure of heat or acid denaturation of L-asparaginase might also have eliminated the postulated activity of impurities.

Accordingly, plasma was taken from a patient prior to and 6 hr after the administration of L-asparaginase and added to the in vitro culture of normal bone marrow of another patient. By assay, the 6-hr plasma sample was shown to contain L-asparaginase, 12 i.u./ml, and by 6 hr impurities such as endotoxin would have been cleared from the plasma (3). Table 1 showed that the pretreatment sample, which contained no L-asparaginase, had no effect on uridine uptake, whereas the 6-hr plasma containing L-asparaginase (0.6 i.u./ml culture) resulted in inhibition which could not be reversed by the addition of L-asparaginase.

These data indicate that the adverse effect of L-asparaginase on normal bone marrow as well as leukemic marrow is due to the enzyme activity per se and not due to any contaminating material.

The details of our clinical results with L-asparaginase will be reported elsewhere. The doses ranged from 6,000 to 50,000 i.u./sq m/day. An initial antileukemic effect in terms of a decrease in the number of circulating leukemic cells occurred in all but 2 of the 22 patients with acute leukemia (Chart 5). This was consistent with the in vitro test wherein L-asparaginase inhibition (L-asparagine dependence) was demonstrated regularly with concentrations of 1.5 i.u./ml. Plasma concentration considerably higher than this were readily maintained during treatment. However, the pretreatment in vitro test did not correlate with the magnitude of the initial antileukemic effect or with the production of remission. The major limitation of L-asparaginase treatment was the relatively transient antileukemic effect. In the majority of patients, blood and organ leukemic cell infiltration recurred, usually within 2 to 6 weeks, in spite of continued treatment.

With one possible exception, bone marrow depression with L-asparaginase did not occur in patients with carcinoma in spite of the demonstrated in vitro L-asparagine dependence. The transient effect of L-asparaginase in acute leukemia and the lack of effect on the normal bone marrow prompted serial in vitro tests (Charts 6 and 7). In a patient with normal bone marrow, the expected decrease in uridine incorporation occurred during L-asparaginase treatment. However, at 7 and particularly 10 days following treatment had been discontinued, uridine incorporation in the presence of L-asparagine in the media had recovered and again there was a marked diminished L-asparagine dependence. In experimental systems, there is evidence that L-asparagine dependence is inversely correlated with the presence of cellular asparagine synthetase (7, 14). Thus, cells resistant to L-asparaginase either have asparagine synthetase initially and/or the synthetase rapidly increases as...
Table 1

Effect of perchloric acid-denatured L-asparaginase and plasma of patients on in vitro test of normal bone marrow

<table>
<thead>
<tr>
<th>Variable in culture</th>
<th>Addition of variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Perchloric acid-denatured L-asparaginase</td>
<td>X</td>
</tr>
<tr>
<td>L-Asparaginase, 0.5 mM</td>
<td>X</td>
</tr>
<tr>
<td>L-Asparaginase, 1.5 i.u./ml</td>
<td>X</td>
</tr>
<tr>
<td>Perchloric acid Urine incorporation</td>
<td>(cpm X 10^6/1.2 X 10^6 cells)</td>
</tr>
<tr>
<td>B. Plasma of patients before and during L-asparaginase treatment</td>
<td>None X</td>
</tr>
<tr>
<td>L-Asparaginase, 0.5 mM</td>
<td>X</td>
</tr>
<tr>
<td>Preplasma without L-asparaginase</td>
<td>X</td>
</tr>
<tr>
<td>6-Hr plasma with L-asparaginase</td>
<td>X</td>
</tr>
<tr>
<td>Urine incorporation</td>
<td>(cpm X 10^6/1.2 X 10^6 cells)</td>
</tr>
</tbody>
</table>

Chart 5. Initial effect of L-asparaginase on circulating leukemic cells in vivo of patients with ALL and AML.

a result of exposure to L-asparaginase, presumably by derepression (14). Accordingly, asparagine synthetase was measured in normal bone marrow cells and in marrows of patients with leukemia (Tables 2 and 3). Enzyme activity was demonstrable prior to treatment (Table 2) in one-half of the normal bone marrows, in one-half the patients with AML, and in none of the patients with ALL. When activities were present, they were considerably lower than that found in normal liver. In 4 patients, 2 with normal marrow and 2 with ALL, asparagine synthetase was measured prior to treatment and 1 time or more during treatment (Table 3). In
Asparagine synthetase activity in patients before L-asparaginase treatment

<table>
<thead>
<tr>
<th>Tissues</th>
<th>No. patients (mmoles product/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal marrow</td>
<td>14</td>
</tr>
<tr>
<td>AML marrow</td>
<td>4</td>
</tr>
<tr>
<td>ALL marrow</td>
<td>4</td>
</tr>
<tr>
<td>Chronic myelogenous</td>
<td>4</td>
</tr>
<tr>
<td>leukeemic marrow</td>
<td>4</td>
</tr>
<tr>
<td>Normal liver</td>
<td>2</td>
</tr>
</tbody>
</table>

Asparagine synthetase activity in patients before and after L-asparaginase treatment

<table>
<thead>
<tr>
<th>Marrows</th>
<th>No. patients</th>
<th>Pretreatment</th>
<th>Posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ALL</td>
<td>2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

It may be concluded that the major limitation in the effectiveness of L-asparaginase in patients with acute leukemia is the rapid development of clinical resistance to L-asparaginase. Serial in vitro tests applied to patients with acute leukemia and to patients with normal marrow undergoing L-asparaginase treatment indicate that L-asparaginase dependence markedly decreases as a result of L-asparaginase treatment. There is evidence that L-asparaginase dependence at a cellular level depends upon the initial concentration of asparagine synthetase and/or on an increase in asparagine synthetase following exposure to L-asparaginase. In the present report, asparagine synthetase was present in very low concentration in one-half of the patients with normal marrows, one-half of the patients with AML, and none of 4 patients with ALL. Following treatment in 2 patients, an increase in asparagine synthetase occurred. Due to the difficulty in obtaining even the minimum amount of tissues required for the enzyme determination initially and repeatedly from human bone marrow, these studies are still in preliminary stage. Recently, a 10-fold increase in sensitivity for the enzyme assay has been developed, and it should partially overcome this difficulty. A careful study is now being continued. These studies indicate that neither the in vitro test nor the determination of asparagine synthetase prior to L-asparaginase treatment will aid in predicting response in vivo. Further studies will be necessary to establish conclusively that L-asparaginase dependence as measured by the in vitro test decreases, that asparagine synthetase activity increases following treatment with L-asparaginase, and that the diminished L-asparaginase dependence results from an increase in asparagine synthetase.

DISCUSSION

The in vitro test for L-asparaginase dependence has correlated closely with the effect of L-asparaginase treatment in experimental in vivo tumor systems (15). In the clinic, we have found the in vitro test to be almost invariably positive in patients with acute leukemia. This is consistent with the observation that almost all patients show an antileukemic effect. Thus, there is a marked reduction and, in some patients, complete elimination of leukemic cells from the peripheral blood, frequently associated with a decrease in organ infiltration (Chart 5). Approximately 50% of patients with ALL and 10 to 20% of patients with AML achieved remission. In our studies, there was no correlation between the magnitude of L-asparaginase dependence as measured by the pretreatment in vitro tests and the ultimate development of remission. The normal bone marrow also exhibited L-asparaginase dependence as evidenced by in vitro tests. This observation further compromises the effectiveness of the in vitro test in predicting in vivo response to L-asparaginase.
Other mechanisms for the development of resistance to L-asparaginase have been described.

An increasing rate of disappearance of the enzyme from the plasma as a result of immunological mechanisms may occur (2). For L-asparagine to enter protein it must be activated and combined to tRNA through the enzyme aminoacyl ribonucleic acid synthetase. An increase in this enzyme might result in an increase in the efficiency of utilization of L-asparagine and thus diminish L-asparagine dependence (R. Gallo, personal communication). Finally, it is possible that L-asparaginase resistance may result from a marked increase in L-asparagine production by the liver and its distribution to organs dependent upon exogenous sources of L-asparagine.

The normal bone marrow, both in experimental systems and in man, is not known to be adversely affected by L-asparaginase treatment. Our in vitro studies indicated that normal bone marrow is adversely affected by L-asparaginase. The possibility that contaminating materials in the L-asparaginase preparation might be responsible for this in vitro inhibition has been excluded (Table 1). We have recently studied the effect of crystalline L-asparaginase on the in vitro test. It also inhibits the uridine incorporation of normal bone marrow. As with acute leukemic cells, serial studies of the in vitro test and of asparagine synthetase indicate that L-asparaginase dependence on a normal bone marrow decreases rapidly. Thus, the effect of L-asparaginase on a normal bone marrow is not of sufficient magnitude and particularly of sufficient duration to produce a functional marrow deficit. The increase in asparagine synthetase in the normal bone marrow following exposure to L-asparaginase is consistent with the increase that occurs in certain normal organs such as the spleen in experimental systems (14).

It was initially hoped that L-asparaginase dependence would be a unique quality to certain neoplastic cells and would not obtain for normal cells. This, unfortunately, has not held up in the clinic where L-asparaginase has been found to inhibit, in addition to the normal bone marrow cells, lymphocyte blastogenesis in vitro (12) and immune response in vivo (E. Hersh, personal communication) and also to inhibit the production of certain normal proteins such as insulin and fibrinogen.

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I-Asparagine Requirement and the Effect of I-Asparaginase on the Normal and Leukemic Human Bone Marrow

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