In Vitro Test for Sensitivity of Leukemic Cells to L-Asparaginase

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SUMMARY

A method is proposed for measuring the cytocidal effect of L-asparaginase on leukemic blood cells as a possible means of screening patients for L-asparaginase therapy. On the average, L-asparaginase was more toxic to blood lymphocytes from patients with chronic lymphocytic leukemia than to lymphocytes from normal blood. A positive sensitivity test was defined as one in which 65% of lymphocytes were killed in 7 days by 0.17 i.u./ml of L-asparaginase. The sensitivity tests were positive in 25 tests on 37 leukemic patients; they were positive in none of the tests on 35 persons with normal hemograms. In 12 leukemic patients with negative sensitivity tests, the disease was in remission in three patients and was in an early stage in one case. Seventeen repeat tests within 5 weeks on 10 patients showed good reproducibility of results. L-Asparaginase was toxic to blood cells of three patients with acute lymphoblastic and myeloblastic leukemia but had relatively little toxicity to blood cells of five other patients with acute leukemia. Lymphocytes from chronic lymphocytic leukemia were usually sensitive to 0.17 i.u./ml, cells from two patients with acute lymphocytic leukemia were sensitive to 0.017 i.u./ml, and cells from the highly L-asparaginase-sensitive mouse lymphoma 6C3HED were sensitive to 0.0017 i.u./ml. In contrast, cells from the in vivo-resistant form of 6C3HED were resistant in vitro to 0.17 i.u./ml.

INTRODUCTION

L-Asparaginase has been tried on an experimental basis for the treatment of patients with acute leukemia and has produced remissions in some patients. (1–3, 5, 9, 10, 13, 19). The enzyme has also proved to be an effective therapeutic agent for some transplantable mouse lymphomas (4).

In view of the variability of the sensitivity of patients to L-asparaginase and the toxic reactions in some patients treated with this reagent (9, 12), it seems necessary to have a suitable test for the selection of patients for treatment. Several in vitro tests have been proposed for measuring the sensitivity of the blood and bone marrow cells of a leukemic patient to L-asparaginase. One test is the measurement of the uptake of uridine or valine into RNA or protein of the leukemic cells incubated with L-asparaginase (10, 11). Another test is the estimation of asparagine synthetase activity of the patient’s blood cells or bone marrow (11). Schrek et al. (17) measured the cytocidal effect of L-asparaginase on blood cells of patients with leukemia. In the present study, the cytocidal test was evaluated by determining the reproducibility of the findings on repeat tests and the variability of the findings during a prolonged observation period of the patient.

MATERIALS AND METHODS

Sensitivity tests were done on blood lymphocytes from 37 patients with chronic lymphocytic or lymphosarcoma cell leukemia, 8 patients with acute leukemia, and 35 persons with normal hemograms.

The in vitro methods used in this laboratory have been described in detail previously (15). In brief, 10 to 20 ml of heparinized blood were obtained from a normal or leukemic patient, and the red blood cells were allowed to sediment for 1 to 2 hr at 37°. The supernatant plasma was centrifuged, and the cells were washed and resuspended in a medium consisting of equal parts of normal human serum and Fischer’s tissue culture medium which contained 10 mg of L-asparaginase/liter. The suspensions were purified by allowing adherence of granulocytes and monocytes to the glass bottom of T-flasks. The nucleted cells in the purified suspensions were 90 to 100% lymphocytes. The suspensions were diluted to give 500 to 1000 cells/mm. Aliquots of the cellular suspensions with and without reagent were distributed in 1-ml amounts in tightly stoppered small test tubes and incubated for 2 to 7 days.

Escherichia coli extracts containing L-asparaginase were obtained from Worthington Biochemical Co., Freehold, N.J. The enzyme was assayed as described previously (7). The extracts contained L-glutaminase activity at 2% of the level of L-asparaginase activity.

For viable cell counts before and after incubation, 0.2 ml of suspension was transferred to a slide chamber composed of 2 cover slips (45 x 50 mm) separated by a metal plate 1 mm thick with a central hole 38 mm in diameter. The average of counts from 3 test tubes was used for each determination. The cells in the slide chamber were examined with an inverted phase-contrast microscope and counted in an area 10 x 0.04 mm. The initial count in a chamber was 200 to 300 viable lymphocytes. Viable lymphocytes were readily differentiated from dead cells by their distinct cytological features, including

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Chart 1. The percentage of effect of 0.17 and 1.7 i.u./ml of L-asparaginase on normal and leukemic lymphocytes incubated for 2 to 7 days with the reagent.

The presence of a distinct nuclear wall, chromatin and nucleolar masses, and irregularities in the shape of the nucleus and cell. The percentage of lymphocytes surviving incubation was based on the number of viable lymphocytes in the suspension before incubation. The percentage of cytotoxic effect of L-asparaginase was calculated by the formula:

\[ \frac{1 - (A/B)}{100} \]

where \( A \) is the viable lymphocytes/cu mm in the incubated suspension with reagent and \( B \) is the viable lymphocytes/cu mm in incubated suspension without reagent.

RESULTS

In a study of the rapidity of the reaction of L-asparaginase on cells, blood lymphocytes from normal individuals and from patients with chronic lymphocytic leukemia were incubated with 0.17 and 1.7 i.u./ml of L-asparaginase for 2, 5, and 7 days. The number of surviving cells was counted before and after incubation. The percentage of cytotoxic effect based on the number of cells in the incubated control tubes is presented in Chart 1. It is seen that large doses of L-asparaginase acted rapidly on the leukemic lymphocytes, while the enzyme had a delayed effect on normal lymphocytes. Statistically significant differences were observed between the sensitivity of normal and leukemic lymphocytes both at 0.17 and 1.7 i.u./ml and after 2, 5, and 7 days of incubation. The maximum difference between normal and leukemic lymphocytes was observed with cells incubated for 2 days with 1.7 i.u./ml.

Dose-effect curves were obtained for the sensitivity of normal and leukemic lymphocytes incubated for 7 days. On the average, 0.05 i.u./ml of enzyme produced a significant difference in sensitivity.

The sensitivity of lymphocytes from individual leukemic patients and normal persons is given in Table 1. On incubation with 1.7 i.u./ml for 2 days, the reagent produced less than 30% effect on lymphocytes from all 11 normal persons and produced more than 40% effect on lymphocytes from all 18 leukemic patients.

When 0.17 i.u./ml of L-asparaginase was incubated with cells for 7 days, the reagent killed 90 to 100% of the lymphocytes from 16 out of 37 leukemic patients. However, the distribution curve appeared to be bi- or trimodal, and at least 11 leukemic patients had lymphocytes with the same sensitivity as normal persons.

If an effect of 65% or more is defined as a positive test for sensitivity and less than 65%, is defined as a negative test, then 25 of 37 leukemic patients and none of the normal persons gave a positive test. Two patients with negative tests with 0.17 i.u./ml for 7 days were sensitive to 1.7 i.u./ml for 2 days (54 and 85% effect).

To check the reproducibility of the results, the tests were repeated once or twice within 5 weeks on 10 patients with positive tests and 4 patients with negative tests. In 17 repeat tests, the findings were within 20% of the first test, and in no case did a positive test become negative or vice versa during a 5-week testing period.

Nine patients were tested repeatedly during a prolonged period of up to 18 months. Two patients gave consistently negative results and 5 gave consistently positive tests. Only 2 patients showed a change in sensitivity of their lymphocytes.

Table 1

<p>| L-Asparaginase tests on lymphocytes from normal persons and patients with chronic lymphocytic leukemia |
|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Normal persons</th>
<th>Leukemic patients</th>
<th>Normal persons</th>
<th>Leukemic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 i.u./ml for 2 days</td>
<td>0.17 i.u./ml for 7 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–9</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10–19</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>20–29</td>
<td>3</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>30–39</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>40–49</td>
<td>2</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>50–59</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>60–69</td>
<td>4</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>70–79</td>
<td>4</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>80–89</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>90–100</td>
<td>1</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>18</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Source of lymphocytes</th>
<th>Date of test</th>
<th>Leukocytes (cells/ml)</th>
<th>L-Asparaginase 0.17 i.u./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2/14/67</td>
<td>353</td>
<td>100</td>
</tr>
<tr>
<td>Lymph node</td>
<td>3/7/68</td>
<td>13</td>
<td>85</td>
</tr>
</tbody>
</table>

\( \% \) effect in 7 days
from positive to negative. In these 2 patients, the change from a positive to a negative sensitivity test was not associated with a remission of the disease but was possibly associated with an increase in the severity of the disease.

Of particular interest are the clinical histories of the 12 patients whose sensitivity tests were negative. Three of these patients had low leukocyte counts and were in remission. One patient had early leukemia with no prior treatment. Eight patients with negative tests had active disease and 4 of them died 13 to 44 days after the test.

Results on 1 patient were particularly interesting. His blood lymphocytes were tested twice within 13 months, and 1 test was done on cells from a cervical lymph node (Table 2). The blood lymphocytes were sensitive, but the nodal lymphocytes were highly resistant to L-asparaginase. The findings could be interpreted as indicating that there was a higher percentage of resistant cells in the lymph node.

**Acute Leukemia.** The blood cells of 8 patients with acute leukemia were available for testing. The survival of the blood cells in the control suspensions varied considerably, and the cells from only 2 patients survived satisfactorily for 7 days. Therefore, it was necessary to use a variable period of incubation, as shown in Table 3. The cells of only 2 patients were highly sensitive even to 0.017 i.u./ml of L-asparaginase. These 2 patients were diagnosed as having acute lymphoblastic leukemia. The cells of 1 patient (E. C.) with acute myeloblastic leukemia were moderately sensitive to 0.17 i.u./ml of enzyme. The other 5 patients had blood cells resistant to L-asparaginase.

**DISCUSSION**

The objective of the present study was to measure the survival of normal and leukemic lymphocytes incubated for 7 days with and without 0.17 i.u./ml of L-asparaginase. A previous study (16) compared the cytocidal effects of L-asparaginase on human and animal neoplastic cells. It was shown that 0.017 i.u./ml of enzyme produced approximately the same cytocidal effect on cells of Walker rat carcinosarcoma and on cells from acute lymphocytic leukemia (75 and 84%, respectively). In *vivo* therapy produced temporary regression of the rat tumor (8) and temporary remission in some patients with acute lymphocytic leukemia (9). In contrast, the small dose of 0.0017 i.u./ml killed nearly all cells from the highly L-asparaginase-sensitive mouse lymphoma 6C3HED, while 0.17 i.u./ml killed only 50% of cells from a resistant variant of 6C3HED (6). The minimum dose which killed neoplastic cells in *vivo* may be an indication of the *in vivo* sensitivity of an animal tumor or a patient to L-asparaginase therapy.

Several other *in vitro* tests have been proposed for measuring sensitivity of leukemic patients to L-asparaginase. One test, the measurement of asparagine synthetase, was based on the finding that the amount of this enzyme was much higher in the resistant variant of 6C3HED lymphoma than in the sensitive form (14). This finding led to the hypothesis that this enzyme is needed to produce endogenous asparagine and that lack of this enzyme by cells makes them vulnerable to L-asparaginase. The test of Sobin and Kidd (18) is commonly used for measuring the effect of L-asparaginase on the uptake of uridine or leucine by leukemic cells. Ho et al. (11) found that L-asparaginase (1.5 i.u./ml) caused a 42% inhibition in the uptake of radioactive uridine by bone marrow cells from acute lymphocytic leukemia. However, there was a similar inhibition (54%) with normal bone marrow cells.

The tests of L-asparaginase sensitivity measure distinctly different physiological or pharmacological properties of leukemic cells, namely (a) the amount of asparagine synthetase, (b) the inhibiting effect of L-asparaginase on RNA synthesis, and (c) the cytotoxic effect of L-asparaginase. The first 2 tests are based on implied hypotheses on the mechanism of the toxic action of L-asparaginase, but the 3rd test measures directly the cytotoxic activity of the enzyme. A dosage of 1.5 i.u./ml of L-asparaginase was needed to give significant results in the test on uridine uptake (11), while 0.17 i.u./ml was used for the cytocidal test. The smaller dose used in the cytocidal test indicates that this test is more sensitive. The period of incubation was 20 hr for the uridine uptake test (11) and 7 days for the cytocidal test. The shorter period is desirable for a clinical test but measures only early effects, while the cytocidal test measures both immediate and delayed effects. The uridine uptake test failed to demonstrate any significant differences in L-asparaginase sensitivity between bone marrow of normal persons and patients with acute lymphocytic leukemia (11). In contrast, the cytocidal test demonstrated considerable differences in the sensitivity of normal and leukemic lymphocytes.
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In the present study, 0.17 i.u./ml produced a 90% cytocidal effect on blood cells of many patients with chronic lymphocytic leukemia and 2 patients with acute lymphoblastic leukemia. A dose of 0.0017 i.u./ml sufficed to produce a 90% effect on cells of the in vivo-sensitive mouse lymphoma 6C3HED. In contrast, 0.17 i.u./ml had relatively little effect on the in vivo-resistant variant of 6C3HED. The minimal dose of L-asparaginase that produces a 90% cytocidal effect may be used as a measure of the in vitro sensitivity of the cells to the enzyme.

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REFERENCES

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