In Vitro and in Vivo Killing of Acute Lymphoblastic Leukemia Cells by L-Asparaginase

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ABSTRACT

L-Asparaginase (ASNase) is a potent antileukemic enzyme routinely used in the treatment of children with acute lymphoblastic leukemia. As part of investigations of the biological activity of ASNase, we have developed techniques which measure the in vitro and in vivo cell killing ability of ASNase. To study the effect of ASNase on in vitro survival of primary lymphoblasts, bone marrow mononuclear cells obtained from untreated patients with acute lymphoblastic leukemia were cultured with and without ASNase. After 5 days, viable cells were counted using trypan blue exclusion to calculate total cell kill due to ASNase. Propidium iodide exclusion, leukemia cell surface antigens, and flow cytometry were used to determine leukemia cell kill due to ASNase. Comparison of leukemia cell kill and total cell kill showed a direct linear relationship (n = 24, r = 0.7), preferential killing of leukemia cells by ASNase (slope = 0.66), and that use of leukemia cell surface markers yielded a more accurate measurement of leukemia cell killing. ASNase at concentrations from 0.0001 to 0.1 IU/ml had equal effects on extent of leukemia cell killing (P = 0.3 to 0.7), suggesting the absence of a dose response at the ASNase concentrations tested. As a measure of the in vivo response to ASNase treatment, the number of viable bone marrow leukemia cells in the patient prior to and 5 days after treatment with ASNase was measured as the product of (% of rhodamine 123 fluorescent viable cells) x (absolute leukemic infiltrate) and that use of leukemia cell surface markers yielded a more accurate measurement of leukemia cell killing. ASNase at concentrations from 0.0001 to 0.1 IU/ml had equal effects on extent of leukemia cell killing (P = 0.3 to 0.7), suggesting the absence of a dose response at the ASNase concentrations tested. As a measure of the in vivo response to ASNase treatment, the number of viable bone marrow leukemia cells in the patient prior to and 5 days after treatment with ASNase was measured as the product of (% of rhodamine 123 fluorescent viable cells) x (absolute leukemic infiltrate). The change which occurred in the viable leukemic infiltrate was the same for patients whether they received 25,000 or 2,500 IU/m2 of ASNase as a single drug. There was a linear correlation (n = 8, r = 0.9) between in vivo and in vitro leukemia cell killing by ASNase. Thus, the in vitro assay described here can be used to predict in vivo sensitivity to ASNase in acute lymphoblastic leukemia.

INTRODUCTION

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is an enzyme that was first identified as an effective antileukemic agent in human clinical trials of the 1970s (1-4) and currently is a vital component of our antileukemia armamentarium (5). As a single agent, it can induce complete remissions in up to 80% of patients with newly diagnosed acute lymphoblastic leukemia (3) and in 26 to 62% of patients with ALL in relapse (1, 3, 4). Clinical trials demonstrated that the addition of ASNase to the treatment of childhood ALL significantly improved disease-free survival (2). It is now used routinely in the treatment of childhood ALL.

The antileukemic effect of ASNase is postulated to result from the rapid and complete depletion of the circulating pool of asparagine. Cytotoxicity is a result of the inhibition of protein synthesis in cells that are unable to synthesize asparagine for their needs and must rely on circulating asparagine.

The ability to predict the sensitivity of an individual's leukemia cells to the cytotoxic effect of ASNase by an in vitro assay would identify those children who might benefit from this therapy and could have a major impact on therapeutic decisions. We have created a therapeutic regimen which permits us to study the in vivo and in vitro response of ALL to treatment with ASNase. Leukemia cells from previously untreated patients are obtained at diagnosis and 5 days after treatment with a single dose of ASNase. The in vivo and in vitro loss of viable leukemia cells are compared as a measure of in vivo and in vitro response to ASNase. Thus, we can assess an early response to single agent therapy.

The long term therapeutic implication of this study is to determine if early biological responsiveness to ASNase as defined by in vitro or in vivo cytotoxicity is prognostic for long term outcome. In this report, we describe the assay systems used to measure in vivo and in vitro ASNase sensitivity, the effect of dose on both in vivo and in vitro leukemia cell killing and the relationship between in vivo and in vitro killing by this drug.

MATERIALS AND METHODS

Patient Sample Collection

After obtaining informed consent, newly diagnosed children with ALL were treated according to a single treatment protocol. They received ASNase (either 2,500 or 25,000 IU/m² by randomized assignment) as a single i.m. injection on the first day of therapy, designated Day 0. On Day 5, patients received four-agent induction therapy. On Days 0 and 5, 2 ml of bone marrow were aspirated and placed in a heparinized tube. An aliquot of the sample was diluted 1:5 in tissue culture medium with 10% FBS and sent at room temperature by an overnight delivery service from the contributing investigators to the University of Rochester for in vitro studies. The remaining aliquot was sent undiluted to the Dana-Farber Cancer Institute for Rh-123 studies.

Summary of Patient Samples

In Vitro Studies. Day 0 bone marrow specimens with adequate cell number for in vitro studies were successfully obtained from 51 of 71 patients' samples sent. The survival of cells cultured without ASNase was judged adequate for analysis (i.e., >40% cell survival after 4 to 6 days in culture) in 37 of these samples cultured. All of these samples were analyzed. Cell surface marker data were available as a measure of specific leukemia cell killing in samples from 24 patients. Of those patients whose leukemia cells expressed demonstrable cell surface markers, the mononuclear cell fraction contained 70 to 99% marker positive leukemia cells. The cells in 20 samples expressed CALLA (CD10), 2 expressed Leu 1 (CD5), 1 expressed Leu 5 (CD2), and 1 expressed B4 (CD19).

In Vivo Studies. Day 0 and Day 5 rhodamine 123 cell viability studies were performed on 67 patients with newly diagnosed ALL. Absolute...
leukemic infiltrate data from both Day 0 and Day 5 were available at the time of analysis on 24 of these samples. The results of both in vitro and in vivo leukemia cell killing data were available for eight patients studied so far.

Materials

Tissue culture media, all culture media additives, and trypan blue were obtained from Gibco, Grand Island, NY, except serum, which was obtained from Hyclone Laboratories, Inc., Logan, UT. The ASNase was a pharmaceutical grade Ershcherichia coli enzyme (Elspar; Merck Sharp & Dohme, Inc., West Point, PA). Ficoll-Hypaque was purchased from Pharmacia, Inc., Piscataway, NJ. Fluorescent reagents were obtained from Becton Dickinson, Mountain View, CA (CALLA, Leu 5 and Leu 1); Coulter Immunology, Hialeah, FL (B4); Sigma Chemical Co., St. Louis, MO (propidium iodide); Eastman Organic Chemicals, Rochester, NY (Rh 123); and Polysciences, Inc., Warrington, PA (Hoechst 33342). All other materials were obtained from Sigma Chemical Co., unless otherwise indicated.

Leukemia Cell Culture

Bone marrow mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. After washing the interface cells free of Ficoll with DME-F12, the cells were washed twice more in DME-F12 and resuspended in tissue culture medium. These bone marrow mononuclear cells were cultured at 5 x 10^6 cells/ml of DME-F12 containing 10% FBS, 10% horse serum, 25 IU/ml penicillin, 25 ìg/ml streptomycin, and 2 mM supplemental glutamine at 37°C in 100% humidity, 5% CO2, and 5% O2. This reduced O2 culture technique was adapted from that of Dicke et al. (6). Prior to incubation, ASNase was added to the culture medium at the following concentrations: 0.0, 0.0001, 0.001, 0.01, 0.1, and 1.0 IU/ml. These cultures were incubated for time periods varying from 2 to 9 days.

After the designated length of time, an aliquot of the cell suspension was diluted 1:1 with trypan blue, and trypan blue excluding (viable) cells were counted in a hemocytometer. In order to determine whether the cells surviving in cell culture medium were leukemia cells, survival was also measured by determining the percentage of viable (propidium iodide excluding) cells that were CALLA (CD10), Leu-1 (CD5), Leu-5 (CD2), or B4 (CD19) positive, depending on the leukemia cell surface markers identified at diagnosis. The proportion of viable marker positive cells was determined by flow cytometry after staining with the appropriate fluorescein conjugated antibody and propidium iodide.

Leukemia Cell Surface Markers and Flow Cytometry. The lymphoblast samples were resuspended in medium and washed with Puck’s Saline G with 15% FBS. Each sample was then resuspended with 50 ìl of the appropriate fluorescein conjugated antibody (CALLA CD10), Leu-5 (CD2), Leu-1 (CD5), B4 (CD19), or isotype-specific control and incubated at 4°C for 30 min. The cells were washed twice more with Puck’s Saline G with 15% FBS. For viability studies, cells were analyzed without fixation on the same day, after addition of propidium iodide to the sample. If the sample was for initial determination of the presence of antigen on the leukemia cells, the cells were fixed in 1% paraformaldehyde, covered with foil and stored at 4°C until run on the flow cytometer. Ten thousand cells were analyzed on an Epics C flow cytometer (Coulter Corp., Hialeah, FL) using forward angle scatter to gate out debris. Green (525 ± 20 nm bandpass) and red (575 ± 10 nm bandpass) fluorescence were used to enumerate antigen-positive and dead cells, respectively. The 488-nm line of an argon laser at 500 mW was used for excitation.

Effect of Incubation Time. When sufficient mononuclear cells were obtained from an individual patient’s sample, cultures were set up in multiple aliquots and each aliquot was evaluated for cell viability at a different incubation time. In order to determine the effect of incubation time on survival in cultures not treated with ASNase, the survival at various time points was compared using paired t test analyses (i.e., Day 2 survival of an individual sample versus Day 5 survival for that same sample). Therefore, to be included in this analysis, it was necessary to have data from a sample at both time points being examined. The effect of incubation time on the cell killing ability of ASNase (both TCK∞ and LCK∞) was examined in the same manner.

In Vivo Response to Asparaginase

The in vivo response of ASNase treatment was assessed by comparing the number of viable leukemia cells before, and 5 days after, treatment of the patient with a single dose of ASNase. The viability of bone marrow cells was examined utilizing Rh-123 fluorescence (7, 8). Bone marrow biopsies were evaluated for cellularity by one investigator (P. L.), and the percentage of lymphoblasts in the bone marrow aspirate specimen were counted by microscopy of the direct smear. The product of these determinations yields an estimate of the concentration of leukemia cells in the patient (8, 9) and is referred to here as the viable leukemic infiltrate (see below).

Rhodamine 123 Uptake. As previously described (7), the lymphoblast samples were resuspended in RPMI 1640 with 15% FBS and 1 mM glutamine at 1 x 10^6 cells/ml. Hoechst 33342, a DNA fluorescent dye, was added to a final concentration of 5 ìM, and the samples were incubated at 37°C for 1 h. The cells were then stained with 5 µg/ml of Rh-123 for 10 min, washed once with medium, and resuspended in rhodamine-free medium containing 5 µM Hoechst 33342. Cell fluorescence was examined 1 h after Rh-123 exposure.

Cells stained with Rh-123 and Hoechst 33342 were analyzed with an Epics D (Coulter) dual source flow cytometer using 100 mW of excitation at 488 nm for Rh-123 and 10 mW at 366 nm for Hoechst 33342. A total of 1000 cells triggered by Hoechst fluorescence were examined. Human lymphoblast cells growing exponentially were stained with Rh-123 and used to standardize Rh-123 fluorescence.

Calculations

The in vitro effect of ASNase on cells in culture is presented as percentage of total cell kill due to ASNase and percentage of leukemia cell kill due to ASNase. Based on the number of viable cells per ml counted by microscopy with trypan blue exclusion

\[
T^{CK} = 1 - \frac{\text{No. of viable cells/ml (ASNase)}}{\text{No. of viable cells/ml (- ASNase)}} \times 100
\]

Based on the results of flow cytometry and propidium iodide exclusion analysis of the number of viable marker positive cells

\[
L^{CK} = 1 - \frac{\text{No. of viable marker positive cells/ml (+ ASNase)}}{\text{No. of viable marker positive cells/ml (- ASNase)}} \times 100
\]

The in vivo response to asparaginase was measured by analysis of the bone marrow cellularity, percentage of lymphoblasts, and percentage of viable cells (i.e., percentage of Rh-123 positive cells) in the Day 0 and Day 5 bone marrow aspirate specimens. These measurements were used to make the following determinations. The subscripts 0 and 5 were used to designate the results from Day 0 and Day 5 specimens, respectively.

\[
ALI^{0} = \frac{\% \text{ lymphoblasts}}{\% \text{ biopsy cellularity}}
\]

\[
ALI^{5} = \frac{\% \text{ lymphoblasts}}{\% \text{ biopsy cellularity}}
\]

\[
VLI^{0} = \frac{\% \text{ rhodamine positive}}{\% \text{ ALI}^{0}}
\]

\[
VLI^{5} = \frac{\% \text{ rhodamine positive}}{\% \text{ ALI}^{5}}
\]

\[
L^{CK} = \frac{\% \text{ rhodamine positive}}{\% \text{ ALI}^{0}} \times 100
\]

RESULTS

In Vitro Cell Survival. The survival at various time points of both marker positive cells and marker negative cells from leukemic bone marrow in culture without ASNase was examined in order to determine the effect of incubation time. There were no differences in total cell survival with incubation times of 2 to 3 days compared with 4 to 6 days (n = 9, P = 0.7) or 4 to 6 days compared with 7 to 9 days (n = 7, P = 0.1). There was a statistically significant decrease in the total cell survival
after 7 to 9 days of incubation as compared to 2 to 3 days (n = 7, P = 0.02). There were no differences in leukemia cell survival with incubation times of 2 to 3 days compared with 4 to 6 days (n = 4, P = 0.3) or 4 to 6 days compared with 7 to 9 days (n = 7, P = 0.1). Too few samples have been studied for leukemia cell survival at both 2 to 3 days and 7 to 9 days to draw any conclusions about differences between these time periods. Thus, for cells incubated between 2 and 9 days, there appears to be at most a small effect of incubation time on either total cell survival or leukemia cell survival.

To examine the relative survival of normal and leukemia cells in our culture system, the percentage of total cell survival was compared to the percentage of leukemia cell survival for all untreated cultures in which cell survival without ASNase was >95% within 5 min of the addition of ASNase. In the presence of 0.01 IU/ml of ASNase, there was a small difference in total cell kill by ASNase between Days 2 and 3 versus Days 4 to 6 (n = 10, P = 0.05) and Days 2 and 3 versus Days 7 and 9 (n = 7, P = 0.03). When the effect of 0.01 IU/ml of ASNase was measured as LCK+, there was a small difference between Days 2 to 3 versus Days 4 to 6 (n = 4, P = 0.06). Too few samples have been studied for leukemia cell kill at both 2 to 3 days and 7 to 9 days to draw any conclusions about differences between these time periods. Based on these results, it would appear that a minimum of 4 days incubation is necessary in order to measure consistently the cell killing effect of ASNase and that incubation times longer than 4 days do not result in statistically significant killing of additional cells. The data for cells incubated 4, 5, or 6 days were selected for further analysis because this time period was most similar to the in vivo exposure to ASNase that we studied.

Effect of ASNase in Vitro. The initial concentration of asparagine in our culture medium was 50 μM, which is similar to that of normal human plasma (10). As can be seen in Fig. 1, when 0.01, 0.1, or 1.0 IU/ml of ASNase was added to the culture media, the asparagine concentration was reduced by >95% within 5 min of the addition of ASNase. In the presence of the two lowest concentrations of ASNase tested, 0.001 and 0.0001 IU/ml, it took 1 to 6 and 12 to 24 h, respectively, to deplete >95% of the asparagine. Therefore, when cultured in the presence of any of the doses of ASNase tested, the cells were exposed to an environment substantially depleted of asparagine. Although the nadir was reached more slowly with the lowest doses, the exposure to an asparagine depleted environment began within 24 h of the start of incubation.

We examined the cell killing effect of ASNase after culturing the cells for multiple different time periods ranging from 2 to 9 days. As shown in Table 1, there was no statistical difference in either total cell kill or leukemia cell kill measured on Day 5 compared to Day 7. This was true at multiple concentrations of ASNase from 0.0001 to 1.0 IU/ml. In the presence of 0.01 IU/ml of ASNase, there was a small difference in total cell kill by ASNase between Days 2 and 3 versus Days 4 to 6 (n = 10, P = 0.05) and Days 2 and 3 versus Days 7 and 9 (n = 7, P = 0.03). When the effect of 0.01 IU/ml of ASNase was measured as LCK+, there was a small difference between Days 2 to 3 versus Days 4 to 6 (n = 4, P = 0.06). Too few samples have been studied for leukemia cell kill at both 2 to 3 days and 7 to 9 days to draw any conclusions about differences between these time periods. Based on these results, it would appear that a minimum of 4 days incubation is necessary in order to measure consistently the cell killing effect of ASNase and that incubation times longer than 4 days do not result in statistically significant killing of additional cells. The data for cells incubated 4, 5, or 6 days were selected for further analysis because this time period was most similar to the in vivo exposure to ASNase that we studied.

In Vitro TCK versus in Vitro LCK. The relationship between in vitro total cell kill and in vitro leukemia cell kill due to ASNase for 24 patients is shown in Fig. 2. This figure demonstrates the linear relationship between leukemia cell kill and total cell kill by ASNase (r = 0.7). The line calculated by linear regression analysis has a slope of 0.66. Thus, in the majority of patients, the effect of ASNase as measured by total cell kill was

Table 1 Cell kill due to ASNase on day 5 versus day 7

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>n</th>
<th>% of cell kill</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Total cell kill</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0001 IU/ml ASNase</td>
<td>5</td>
<td>21</td>
<td>27.2 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>21</td>
<td>29.8 ± 8.0</td>
</tr>
<tr>
<td>0.001 IU/ml ASNase</td>
<td>5</td>
<td>21</td>
<td>38.6 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>21</td>
<td>33.3 ± 7.7</td>
</tr>
<tr>
<td>1.0 IU/ml ASNase</td>
<td>5</td>
<td>20</td>
<td>43.6 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>20</td>
<td>39.7 ± 10.6</td>
</tr>
<tr>
<td>B. Leukemia cell kill</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00001 IU/ml ASNase</td>
<td>5</td>
<td>13</td>
<td>34.9 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>13</td>
<td>38.3 ± 8.4</td>
</tr>
<tr>
<td>0.01 IU/ml ASNase</td>
<td>5</td>
<td>13</td>
<td>42.1 ± 10.4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>13</td>
<td>41.3 ± 8.1</td>
</tr>
<tr>
<td>1.0 IU/ml ASNase</td>
<td>5</td>
<td>14</td>
<td>54.6 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>14</td>
<td>49.8 ± 9.6</td>
</tr>
</tbody>
</table>

* Values represent the mean ± SE of results for all samples cultured for a given time period.

** Significance determined by paired t test of cell kill at Day 5 versus Day 7.

Bone marrow mononuclear cells were cultured in duplicate with 0.0, 0.0001, 0.01, and 1.0 IU/ml of ASNase. One set of cultures was incubated for 5 days, and a duplicate set was incubated for 7 days. The cell kill due to ASNase (both TCK+ and LCK+) is calculated based on the number of viable cells in the presence of ASNase compared to the number of viable cells in the untreated culture (see "Materials and Methods"). A, the number of remaining viable cells was counted by trypan blue exclusion, and percentage of total cell kill due to ASNase was calculated.

Fig. 1. Effect of time on asparagine depletion. For these experiments, 5 ml of DME-F12 culture media were placed in tubes which already contained 0.056 ml of [U-14C]asparagine (about 50,000 cpm); Amershams Corp., Arlington Heights, IL). Different concentrations of ASNase were added to start the reaction. Each ASNase media mixture was immediately divided into 5 aliquots of 1 ml. An aliquot of each ASNase concentration was placed immediately on ice as the zero incubation point and then extracted with methanol. The remaining aliquots were placed in culture wells and incubated at 37°C, 100% humidity, 5% CO2, and 5% O2. For 6, 12, 24, and 48 h. At the end of the designated time, an aliquot of each ASNase concentration was removed to a glass test tube, placed on ice, and extracted with methanol. The extraction supernatant was saved and frozen at −70° C until all incubations were completed. The samples were lyophilized and resuspended, and asparagine was quantitated after passage over a Bio-Rad AG 1-X8 anion exchange column (0.4 × 3.2), a method modified from Gantt et al. (11). This figure shows recovery of [U-14C]asparagine reported as percentage of control recovery. [U-14C]asparagine was added to the culture media at the time of cell addition. The ratio of [U-14C]asparagine added to the culture media and [U-14C]asparagine quantitated at 5 h was calculated and used to determine the recovery of [U-14C]asparagine. Each point is representative of duplicate experiments.

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significant increase in leukemia cell killing at 1.0 IU/ml when compared with either 0.0001 or 0.1 IU/ml.

Dose Response in Vivo. As a measure of the in vivo antileukemic effect of ASNase therapy, the changes in the viability of aspirated bone marrow mononuclear cells and in the absolute leukemic infiltrate were examined 5 days after a single dose of ASNase. As shown in Fig. 4, there was no difference in the change in percentage of rhodamine positive viable cells for the 12 patients given high dose as compared to the 14 patients given low dose [n = 26, P = 0.96; mean, 22.6% ± 14.6 (SD) versus 21.6% ± 21.8]. The change in absolute leukemic infiltrate was the same for the high dose patients as for the low dose patients (n = 24, P = 0.9; mean, 25.4% ± 28.4 versus 27.4% ± 28.4) (Fig. 5). Also, the LCKₚ for the high dose patients was the same as for the low dose patients (n = 18, P = 0.9; mean, 41.4% ± 48.0 versus 38.8% ± 52.9) (Fig. 6). In order to assure that the results in patients with a minimal response to ASNase would not mask a dose effect apparent only in those patients with some measurable response to ASNase, the samples were subdivided into two groups based on the LCKₚ. Samples were classified as high if the LCKₚ was >40%, and as low if the LCKₚ was <40% (i.e., same criteria as used for in vitro analysis). When analyzed in this manner, there was no difference in LCKₚ for the high dose patients as compared to the low dose patients within either the high group or the low group (P ≥ 0.7). As found for the in vitro leukemia cell kill, the in vivo 5-day response to ASNase appears to be the same at the two doses used.

In Vivo LCK versus in Vitro LCK. For eight patients, complete in vivo and in vitro leukemia cell killing measurements less than leukemia cell kill. This indicates a relatively increased sensitivity of the leukemia cells to the cytocidal activity of ASNase. For subsequent studies we used LCKₚ as the most accurate measure of the antileukemic effect of ASNase.

Dose Response in Vivo. The effect of various concentrations of ASNase on in vitro leukemia cell kill for 20 patients is shown in Fig. 3. For this analysis, patient samples were divided into two groups in order that the results in samples with no measurable effect of ASNase at any concentration would not mask a dose response apparent only in samples with some measurable effect of ASNase. Samples were classified in the “high” group if the mean of the LCKₚ, in the presence of 0.0001, 0.001, 0.01, and 0.1 IU/ml of ASNase was >40% (n = 10); and in the “low” group if this mean was <40% (n = 10). The leukemia cell killing for each ASNase concentration is shown as the mean ± SD for all high group samples (C) and low group samples (O) versus ASNase concentration. Serial paired t tests were performed comparing the LCKₚ, at 0.0001 versus 0.001, 0.0001 versus 0.01, 0.0001 versus 0.1, and 0.0001 versus 1.0 IU/ml for each sample group. The resultant P values are shown.

Fig. 2. Comparison of total cell kill (TCKₑₚ) versus leukemia cell kill (LCKₑₚ) after 5 days incubation with ASNase. Percentage of cell kill was measured by two different methods (see “Materials and Methods”). O, mean of the percentage of cell kill at 4 concentrations of ASNase tested (0.0001, 0.001, 0.01, and 0.1 IU/ml) for a single patient (n = 24). The linear regression line has a slope of 0.66 and a correlation coefficient of 0.7.

Fig. 3. Effect of ASNase concentration on in vitro leukemia cell killing. Bone marrow mononuclear cells obtained from previously untreated patients with ALL (n = 20) were cultured with and without ASNase added to the media, and cell survival was measured after 4 to 6 days (see “Materials and Methods”). Samples were analyzed in 2 groups: the “high” group if the mean of the LCKₑₚ in the presence of 0.0001, 0.001, 0.01, and 0.1 IU/ml of ASNase was >40% (n = 10); and the “low” group if this mean was <40% (n = 10). The leukemia cell killing for each ASNase concentration is shown as the mean ± SD for all high group samples (C) and low group samples (O) versus ASNase concentration. Serial paired t tests were performed comparing the LCKₑₚ, at 0.0001 versus 0.001, 0.0001 versus 0.01, 0.0001 versus 0.1, and 0.0001 versus 1.0 IU/ml for each sample group. The resultant P values are shown.

Fig. 4. In vivo effect of ASNase dose on the percentage of viable cells in bone marrow. The change which occurs in percentage of viable cells present in bone marrow samples obtained prior to and 5 days after treatment of the patient with ASNase is shown as a function of ASNase dose. Change in percentage of viable cells = percentage of Rh-123 positive cells on Day 0 - percentage of Rh-123 positive cells on Day 5. O, patient who received 2,500 IU/m² (n = 14) after treatment of the patient with ASNase is shown as a function of ASNase dose. Change in percentage of viable cells = percentage of Rh-123 positive cells on Day 0 - percentage of Rh-123 positive cells on Day 5. O, patient who received 2,500 IU/m² (n = 14); @, patient who received 25,000 IU/m² (n = 18). Student's t test, P = 0.96.

Fig. 5. In vivo effect of ASNase dose on absolute leukemic infiltrate. The change which occurs in ALI measured in bone marrow samples obtained prior to and 5 days after treatment of the patient with ASNase is shown as a function of ASNase dose. Change in absolute leukemic infiltrate = ALI on Day 0 - ALI on Day 5. O, patient who received 2,500 IU/m² (n = 14); @, patient who received 25,000 IU/m² (n = 10). Student's t test, P = 0.9.
relationship between in vivo and in vitro cell killing.

to a single drug, providing a unique opportunity to study the problem of acquired drug resistance. In vivo as well as in vitro biological responsiveness to this antileukemic agent. The study of ASNase as a single antileukemic agent is followed by a 5-

single agents on killing of leukemia cells. The administration of ASNase to patients with ALL that permits us to study the early effects of the in vivo and in vitro killing of leukemia cells with ASNase.

ASNase on leukemia cell kill is shown in Fig. 7. Linear regression analysis demonstrated a direct linear relationship between leukemia cell kill and total LCKp, at four concentrations of ASNase (0.0001, 0.001, 0.01, and 0.1 lU/ml). Each point (•) represents a single patient result. The line calculated by linear regression analysis has a slope of 0.6 and a correlation coefficient of 0.9.

were available. Comparison of the in vivo and in vitro effects of ASNase on leukemia cell kill is shown in Fig. 7. Linear regression analysis demonstrated a direct linear relationship between the in vivo and in vitro killing of leukemia cells with ASNase (r = 0.9). Thus, both systems appear to be measuring drug sensitivity in a similar manner.

DISCUSSION

Prognostic factors have been very important in the development of current leukemia protocols, which are designed to “tailor” therapy based on relative risk of treatment failure versus risk of long term treatment toxicities. Recently, attention has turned to responsiveness to initial chemotherapy as a prognostic feature. An initial rapid response to chemotherapy may predict a successful outcome (9, 12–14).

We have developed a treatment program for newly diagnosed patients with ALL that permits us to study the early effects of single agents on killing of leukemia cells. The administration of ASNase as a single antileukemic agent is followed by a 5-day period of investigation using laboratory measures of in vivo and in vitro cell killing due to ASNase to learn more about biological responsiveness to this antileukemic agent. The study of newly diagnosed, previously untreated patients obviates the problem of acquired drug resistance. In vivo as well as in vitro measurements of leukemia cell kill are obtained after exposure to a single drug, providing a unique opportunity to study the relationship between in vivo and in vitro cell killing.

Many authors have reported positive correlations between the results of in vitro assays and in vivo chemotherapy in humans (15–19), but the perfection of a practical test has not been achieved. Lack of a clonogenic assay has in the past precluded such studies in ALL. Recently, however, Weisenthal et al. (15–17) have adapted a mass culture nonclonogenic assay technique utilizing a dye exclusion method for measure of tumor cell kill, for use in determining the in vitro chemosensitivity of human tumors. The cell suspension culture assay requires a small number of cells and does not depend on cell growth in culture, which makes it possible to assay ALL cells that do not grow well in culture. The majority of patients studied were treated with drug combinations rather than with single agents, and successful remission induction was used as a measure of drug response, so they were unable to make valid in vitro versus in vivo correlations.

Our data show a strong correlation between in vivo and in vitro ALL cytotoxicity of ASNase. Thus, the in vitro assay system in which ASNase concentration remains constant for the 5 days can be used to predict in vivo sensitivity to ASNase even though the serum ASNase concentration decreases 2- to 3-fold during the 5-day period of observation (20). This result allows valid comparison between in vitro and in vivo measures of ASNase cytotoxicity.

We measured both total cell killing and leukemia cell killing in the samples using leukemia cell surface antigens in order to discriminate leukemic cells from normal cells. There was a direct linear relationship between leukemia cell kill and total cell kill by ASNase. Based on the fact that the slope of the line was <1, we conclude that ASNase preferentially kills leukemia cells. This is consistent with the decreased sensitivity of normal bone marrow to ASNase. Further analysis therefore required the use of cell surface marker studies for accurate measure of leukemic cell killing.

The possibility of a dose response to ASNase was suggested by the results of a clinical trial reported by Ertel et al. (4). Such a dose effect had not been noted previously by other investigators. In in vitro studies, we did not observe any such dose effect of ASNase at the doses tested. In vitro killing of leukemia cells was independent of concentration from 0.0001 to 0.1 lU/ml of ASNase. This result is consistent with our findings that asparagine is markedly diminished in culture medium by addition of low concentrations of ASNase. The increase in cytocidal activity at 1.0 lU/ml observed among the high group suggests that there may be an in vitro mechanism of ASNase cell killing other than by asparagine depletion. Studies are under way in our laboratory to address this possibility. As expected from the known pharmacology of ASNase and the lack of an in vitro dose response for this drug, killing of leukemia cells in vivo was found to be the same at 25,000 and 2,500 IU/m2 of ASNase. Thus, as for the in vitro data, the in vivo 5-day response rate to ASNase appears to be the same at the doses tested. We have shown that, using concentrations of ASNase of ≥0.0001 IU/ml, we can deplete asparagine in our tissue culture medium. Because circulating asparagine may be continually produced in vivo, a better understanding of the role of asparagine depletion as well as the pharmacological effect of ASNase in vivo will be necessary to define the optimal dosage and schedule of ASNase.

These measurements of in vivo and in vitro cell killing have not been correlated with the long term outcome of the patients yet, and therefore a meaningful definition of response has not been established. As seen in Figs. 2 and 4 to 6, leukemia cells from certain patients were not killed by ASNase either in vivo or in vitro. The determination of whether those patients whose leukemia cells are not killed or are killed less rapidly by ASNase...
either in vivo or in vitro may be at significantly higher risk of relapse is part of ongoing investigations.

Utilizing this system, some, but not all, patients with ALL demonstrate a sensitivity of their lymphoblasts to ASNase in vivo as well as in vitro. This assay system may be helpful in defining biological responsiveness to ASNase. Future analyses of the data obtained will determine if a lack of a response to ASNase is predictive of ultimate recurrence of leukemia and the need for alternate therapies. The combined in vitro and in vivo investigations on ASNase outlined here may serve as a model for study of other drugs in the treatment of leukemia.

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In Vitro and in Vivo Killing of Acute Lymphoblastic Leukemia Cells by l-Asparaginase

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