The Effects of Phenylalanine Ammonia-Lyase on Leukemic Lymphocytes in Vitro

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SUMMARY

Phenylalanine ammonia-lyase, which irreversibly deaminates phenylalanine and tyrosine, markedly inhibited the growth of human leukemic and murine L5178Y lymphoblasts in vitro but had a negligible effect on resting (nondividing) normal lymphocytes. These studies also demonstrated that phenylalanine ammonia-lyase inhibited DNA synthesis in leukemic cells at least as effectively as did asparaginase. Furthermore, neither cinnamic acid, coumaric acid and ammonia, the products of phenylalanine and tyrosine deamination, nor inactivated phenylalanine ammonia-lyase affected the growth of the lymphoblasts. These results suggest that phenylalanine ammonia-lyase inhibited cell division by depleting these rapidly dividing cells of phenylalanine and tyrosine.

INTRODUCTION

Currently, the nutritional regulation of tumor growth either by control of amino acid intake or by the use of enzymes is receiving attention in a number of laboratories (7, 13, 14, 17). Many enzymes catalyze the essentially irreversible degradation of amino acids. This quality makes these enzymes potentially useful as agents that deplete specific amino acids in vitro or in vivo. The dietary control of tumor growth by manipulation of the nonessential amino acids is difficult, since by definition the organism synthesizes these metabolites. Because of the specialization of tissues in complex animals, however, biosynthesis frequently takes place primarily at a restricted number of organ sites. An implication is that certain tissues, such as neoplastic ones, may not have the ability to synthesize certain of the nonessential amino acids in adequate amounts. This has proven to be the case with certain leukemias in which there is a dependence upon plasma concentrations of asparagine (17) and serine (21). Consequently, asparaginase is effective in causing suppressions of several sarcomas (15) and leukemias in mice (3) and of lymphatic leukemias in dogs (18) and humans (10).

In contrast, essential amino acids may be restricted either by dietary manipulation or by the use of specific enzymes. Lorincz and Kuttner (13, 14), utilizing phenylalanine dietary restriction as a treatment for patients with a variety of solid tumors, have reported some provisional successes. In several cases definite tumor regression was observed. A minimal level of phenylalanine was found which had no apparent adverse effects upon the host while causing tumor inhibition. These promising results were obtained with metastatic vulvar malignant melanoma, severe chronic Hodgkin's disease, and advanced pelvic squamous cell carcinoma. A period of 4 to 6 weeks of deficient diet, however, was required for the significant lowering of serum amino acid concentrations. Alternatively, the administration of phenylalanine ammonia-lyase, which specifically deaminates phenylalanine to cinnamic acid and tyrosine to coumaric acid, would effect a rapid decrease in the concentration of these amino acids in the serum.

The availability of leukocytes from patients with acute lymphoblastic leukemia and from a murine lymphoblastic cell line that may be maintained in culture provides a unique means for determining the effects of this enzyme on neoplastic cell division. Studies described below indicate the ability of phenylalanine ammonia-lyase to inhibit the growth of human leukemic lymphocytes and murine lymphoblasts in vitro.

MATERIALS AND METHODS

Materials. Thymidine-³H (specific activity, 2 Ci/m mole) and L-tyrosine-³H (specific activity, 42.9 Ci/m mole) were obtained from New England Nuclear, Boston, Mass. L-Phenylalanine-U-¹⁴C (specific activity, 12.6 mCi/m mole) was obtained from Amersham-Searle Corp., Des Plaines, Ill. Leukopaks were a product of Fenwal Laboratories, Morton Grove, Ill. All media and supplements were obtained from Grand Island Biological Co., Grand Island, N. Y. Cinnamic acid and coumaric acid were obtained from Sigma Chemical Co., St. Louis, Mo. PHA was purchased from Difco Laboratories, Detroit, Mich., Rhodotorula glutinis was purchased from P-L Biochemicals Inc., Milwaukee, Wis., and all other chemicals were reagent grade.

Cell Cultures. Leukocytes were obtained from 5 patients with acute lymphoblastic leukemia and maintained in 2-oz prescription bottles at a concentration of 10⁷ cells/5 ml of modified McCoy's 5A medium supplemented with 10% fetal calf serum at 37° in an atmosphere containing 5% CO₂ in balanced air. These cells were treated with different concentrations of phenylalanine ammonia-lyase or asparaginase and their effects on DNA synthesis were determined by measuring the incorporation of labeled thymidine into DNA (1). Radioactivity was determined in

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2 The abbreviations used are: U, the uniformly labeled compound; PHA, phytohemagglutinin; FHS, Fischer's medium plus 10% horse serum.

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Bray's solution (4) as indicated previously, and samples were analyzed for DNA content by the procedure of Burton (6). This technique, rather than a simpler one involving cell counts, was used to estimate cell growth because of the heterogeneous cell population that exists in leukocytes obtained from patients and because these cells (leukemic and PHA-stimulated normal lymphocytes) tend to agglutinate.

Normal lymphocytes were obtained from 6 healthy donors and separated from polymorphonuclear leukocytes on Leukopak or glass wool by the procedure indicated previously (1). The effect of phenylalanine ammonia-lyase treatment on these cells while in the nondividing state was determined by incubating the cells (10⁷ cells/5 ml modified McCoy's 5A medium) with different concentrations of enzyme for 1 day. The cells were separated from the medium by centrifugation (300 X g) and washed twice with fresh medium to remove the enzyme. The cells were then suspended in fresh medium and stimulated to divide with PHA (40 µg), and DNA synthesis and DNA content were determined as indicated previously.

The effect of phenylalanine ammonia-lyase treatment on normal dividing lymphocytes was also determined. Normal resting lymphocytes (10⁷ cells/5 ml modified McCoy's 5A medium) were stimulated to divide by treatment with PHA (40 µg). The cells were then treated with different concentrations of phenylalanine ammonia-lyase, and both DNA synthesis and DNA content were determined as indicated previously.

Murine leukemic lymphoblasts (L5178Y) were maintained in vitro in Fischer's media (8). Cell growth was readily assessed by measuring cell numbers. These were determined either with a hemacytometer or with a Coulter Counter, Model B, and the generation time obtained for this line was 11 ± 1 hr. Following treatment with different concentrations of enzyme, these lymphoblasts (1.5 X 10⁶ cells/15 ml FHS) were counted and examined microscopically for cell viability with the Erythrosin B dye exclusion test (20).

All analyses were done in duplicate, and each experiment was repeated at least twice.

Preparation of Phenylalanine Ammonia-Lyase. Phenylalanine ammonia-lyase was prepared by the method of Hodgins (12) from the yeast Rhodotorula glutinis. A unit of phenylalanine ammonia-lyase is defined as that amount of enzyme that produces 1 nmole of cinnamic acid per min at 30°. The reaction mixture (1 ml) consisted of 0.833 mM L-phenylalanine, 0.1 M Tris-Cl (pH 8.5), and 10 µl of enzyme. Three separate preparations gave enzymes with specific activities of 0.48, 0.54, and 1.0 unit/mg protein, respectively.

The activity of highly purified phenylalanine ammonia-lyase varied in specific activity from 0.5 to 1.2 units/mg protein (12). This enzyme, when stored aseptically at 4°, was stable for approximately 1 month without significant loss of activity.

Stability of Phenylalanine Ammonia-Lyase in Medium. The activity of phenylalanine ammonia-lyase in FHS with and without cells was determined at various time intervals throughout the incubation period. Aliquots (10 µl) of the medium were assayed for enzyme activity by the method of Hodgins (12) as indicated previously.

Preparation of Cinnamic Acid-14C and Coumaric Acid-3H. Cinnamic acid-14C and coumaric acid-3,5-3H were prepared enzymatically. A reaction mixture containing L-phenylalanine-14C (50 µCi) in 0.05 M Tris-Cl, pH 8.5 (1 ml) was incubated with 1.2 units phenylalanine ammonia-lyase for 64 hr. Another mixture containing L-tyrosine-3,5-3H (1 mCi) was incubated with 0.6 units phenylalanine ammonia-lyase for 52 hr. In the latter reaction, unlabeled L-tyrosine (1 mg) was added to provide additional substrate. The reactions were stopped by the addition of concentrated HCl (0.1 ml), cooled to 4° for 30 min, and centrifuged at 23,500 X g for 5 min. Each acidified supernatant (0.5 ml) was diluted to 1.5 ml with distilled water and extracted with diethyl ether (3 ml). The aqueous layer was reextracted 1 more time with ether. The 2 ether extracts were combined and reextracted with 0.1 N sodium hydroxide (2 ml). This procedure is a modification of the method of Uchiyama et al. (24).

Cinnamic acid-U-14C and coumaric acid-3-3H were separated by descending paper chromatography in l-butanol:acetic acid:water (450:50:125) (2). A Vanguard paper chromatogram scanner was used to locate the radioactivity on the paper, and UV light was used to visualize the cinnamic and coumaric acids. Each preparation contained only 1 radioactive peak, and these peaks corresponded to authentic cinnamic acid and coumaric acid, respectively.

Determination of Rate of Conversion of L-Phenylalanine and L-Tyrosine to Cinnamic Acid and Coumaric Acid by Phenylalanine Ammonia-Lyase. FHS (15 ml) either with or without the addition of leukemic cells was treated with phenylalanine ammonia-lyase at various concentrations. The rates of deamination of phenylalanine and tyrosine were determined spectrophotometrically by measuring the formation of cinnamic acid and coumaric acid following their extraction from the media by a modified procedure based upon that of Uchiyama et al. (24). The efficiency of extraction was measured by adding the prepared radioactive products, cinnamic acid-U-14C (6,600 dpm) and coumaric acid-3,5-3H (20,700 dpm), to the media (0.5 ml) prior to extraction. These radioactive standards did not contribute significantly to the final absorbances of the unlabeled cinnamic and coumaric acids (<0.05 A). The concentrations of cinnamic and coumaric acid produced by treatment with phenylalanine ammonia-lyase were measured spectrophotometrically at 268 and 333 mµ, respectively. An additional correction was used to compensate for the coumaric acid absorbance at 268 mµ (24). The concentrations of cinnamic and coumaric acids were then corrected for the efficiency of extraction to determine the actual amounts of each produced.

The total phenylalanine and tyrosine concentrations in FHS were determined independently by the enzymatic procedure described below and by fluorometry (16, 25). In the enzymatic determination, L-phenylalanine-14C (135,000 dpm) and L-tyrosine-3,5-3H (131,000 dpm) were added to 1 ml FHS. The pH of the medium was adjusted to 8.5 with 1 N NaOH for optimal enzyme activity. The medium was then incubated with 5.0 units phenylalanine ammonia-lyase (1 ml) for approximately 65 hr. The reaction was terminated, and cinnamic and coumaric acids were determined as described above. Both methods gave essentially the same values for phenylalanine (55 µg/ml) and tyrosine (115 µg/ml) in FHS.
Phenylalanine Ammonia-Lyase and Leukemic Lymphocytes

The effect of phenylalanine ammonia-lyase on DNA synthesis in leukocytes from a patient with acute lymphoblastic leukemia was determined. Approximately $10^7$ cells/5 ml McCoy's 5A media were incubated at 37°C in an atmosphere of 5% CO₂ in balanced air for the indicated times. On each day, thymidine-$^3$H (2 $\mu$Ci/ml) was added 6 hr prior to the termination of the experiment, and incorporation of this precursor into DNA and DNA content were assayed as indicated in “Materials and Methods.” Curve A, untreated acute lymphoblastic leukemia cultures; Curves B, C, and D, cells that were treated with 0.025, 0.125, and 0.250 unit of phenylalanine ammonia-lyase, respectively.

RESULTS

The effect of phenylalanine ammonia-lyase on leukocytes obtained from one of the patients under study with acute lymphoblastic leukemia is shown in Chart 1. This patient had a white blood cell count of 237,000/cu mm, and a differential count revealed the presence of 99% lymphoblasts in the peripheral blood. Curve A represents untreated acute lymphoblastic leukemia cultures. These cells exhibit appreciable DNA synthesis during the 1st 6 hr in culture (1,200 dpm of thymidine incorporated per $\mu$g of DNA) and the rate increased approximately 3-fold during the next 4 days. Curves B, C, and D indicate cells ($10^7$) that were treated with 0.025, 0.125, and 0.250 unit of phenylalanine ammonia-lyase, respectively. The results show that 0.025 unit of phenylalanine ammonia-lyase had little effect on DNA synthesis while additions of 0.125 and 0.250 unit resulted in 65 and 80% inhibition, respectively, at Day 4. Although some variation in the extent of DNA synthesis occurred in lymphocytes from the 5 patients studied, the pattern of inhibition obtained with phenylalanine ammonia-lyase was similar in all cases as exemplified here.

A similar study was performed to determine the effects of asparaginase on DNA synthesis in acute lymphoblastic leukemia leukocytes (Chart 2). Curve A represents the untreated acute lymphoblastic leukemia lymphocytes; Curves B, C, and D, cells that were treated with 0.1, 1.0, and 10.0 units of asparaginase, respectively. The results, which are generally consistent with those obtained by others (5, 11, 17, 19), demonstrated that DNA synthesis in acute lymphoblastic leukemia leukocytes is markedly reduced following treatment with asparaginase. When Charts 1 and 2 are compared, phenylalanine ammonia-lyase and asparaginase appear to be equally effective in inhibiting DNA synthesis in acute lymphoblastic leukemia cultures, but phenylalanine ammonia-lyase is at least twice as effective as asparaginase when the enzymes are compared on the basis of units of activity at the pH of the medium (pH 7.4).

In other experiments, it was found that neither cinnamic acid, nor coumaric acid and NH₃, the products of phenylalanine and tyrosine deamination, nor inactivated phenylalanine ammonia-lyase (the catalytic site was inactivated by treatment with sodium borohydride) (12) inhibited DNA synthesis in cultures containing dividing cells. These results strongly suggest that phenylalanine ammonia-lyase exerts its effect by depriving the leukemic lymphocytes of phenylalanine and tyrosine.

The effect of phenylalanine ammonia-lyase treatment on normal lymphocytes in the nondividing state was determined by pretreating these cells with enzyme for 1 day, changing the media to remove the enzyme and measuring the proliferative capacity as a function of PHA stimulation (Table 1, Experiment A). Pretreatment of these normal cells in the nondividing state with relatively high concentrations of enzyme (0.500 unit) had essentially no effect on the DNA synthesis resulting from subsequent PHA stimulation.

The effect of phenylalanine ammonia-lyase treatment on
small population of viable cells. Consequently, phenylalanine cell lysis, was present in the medium that could maintain a incomplete conversion of phenylalanine to cinnamic acid or 98 ± 2%. In contrast to these growth patterns, the control had a doubling time of 10.5 hr with a cell viability of least 1 month. In addition, the viability decreased within 24 hr and less than 2% of the original population was viable after treatment for 3 days with phenylalanine ammonia-lyase. Upon further studies, it was found that the viability decreased within 24 hr and less than 2% of the original population was viable after treatment for 3 days with phenylalanine ammonia-lyase. Additional studies demonstrated that cell growth did not resume in the presence of phenylalanine ammonia-lyase for at least 1 month. In contrast to these growth patterns, the control had a doubling time of 10.5 hr with a cell viability of 98 ± 2%.

Further experiments were performed to determine whether or not a small amount of phenylalanine, left by either incomplete conversion of phenylalanine to cinnamic acid or cell lysis, was present in the medium that could maintain a small population of viable cells. Consequently, phenylalanine and tyrosine concentrations were measured following treatment of the medium with phenylalanine ammonia-lyase in the absence and presence of L5178Y lymphoblasts. The results of this study (Chart 4) show that there was 100% conversion of phenylalanine to cinnamic acid in the medium with or without cells by approximately 45 hr after phenylalanine ammonia-lyase addition. Consistent with this observation was the finding that the enzymatic activity of phenylalanine ammonia-lyase in culture media was constant for 71 hr, and in other experiments it was shown to be constant for at least 2 weeks. Although phenylalanine was completely converted to cinnamic acid, phenylalanine ammonia-lyase deaminates tyrosine more slowly with only 75% of the tyrosine converted to coumaric acid during the 71-hr incubation with the enzyme.

**DISCUSSION**

The results of these studies demonstrate that phenylalanine ammonia-lyase effectively inhibited DNA synthesis in leukocytes obtained from patients with acute lymphoblastic leukemia and caused a rapid cessation of cell growth of L5178Y murine lymphoblasts with the eventual loss of cell viability and concomitant cell death. Since the murine lymphoblasts divide rapidly ($T_D = 11 ± 1$ hr), approximately 3.25 units of phenylalanine ammonia-lyase were required to cause a rapid cessation of growth. Lower concentrations of normal lymphocytes in the dividing state was also determined by treating these cells with enzyme after PHA addition (Table 1, Experiment B). Phenylalanine ammonia-lyase caused both a delay in the onset of DNA synthesis and marked inhibition. Additional studies demonstrated that DNA synthesis remained inhibited at later times (Days 5, 6, and 8). When the effect of phenylalanine ammonia-lyase on nondividing and dividing lymphocytes was compared under identical conditions (0.500 unit of phenylalanine ammonia-lyase), DNA synthesis was 4-fold less by Day 4 in the lymphocyte cultures which were treated with enzyme after PHA stimulation.

Chart 3 shows the effects of phenylalanine ammonia-lyase on the murine lymphoblastic line, L5178Y. Treatment of these lymphoblasts with phenylalanine ammonia-lyase (3.25 units) resulted in a rapid inhibition of cell growth (within 24 hr). Treatment with higher concentrations of phenylalanine ammonia-lyase (5.25 and 7.50 units) did not reduce the cell count more than 50% of the initial inoculum. When these cells were examined microscopically after Erythrosin B staining (20), however, it was found that the viability decreased within 24 hr and less than 2% of the original population was viable after treatment for 3 days with phenylalanine ammonia-lyase. Additional studies demonstrated that cell growth did not resume in the presence of phenylalanine ammonia-lyase for at least 1 month. In contrast to these growth patterns, the control had a doubling time of 10.5 hr with a cell viability of 98 ± 2%.

Further experiments were performed to determine whether or not a small amount of phenylalanine, left by either incomplete conversion of phenylalanine to cinnamic acid or cell lysis, was present in the medium that could maintain a small population of viable cells. Consequently, phenylalanine

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Enzyme concentration (Total units)</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
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</thead>
<tbody>
<tr>
<td>A. Pretreatment with phenylalanine ammonia-lyase before PHA</td>
<td>0.112&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26,100</td>
<td>40,700</td>
<td>26,000</td>
</tr>
<tr>
<td>B. Posttreatment with phenylalanine ammonia-lyase after PHA</td>
<td>0.225&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30,000</td>
<td>37,500</td>
<td>21,000</td>
</tr>
</tbody>
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<sup>a</sup> Cells from a different donor.
and Ryan and Lorincz (23) also found that PHA-stimulated normal cells was inhibited by phenylalanine less rapidly than with higher concentrations of enzyme. Inhibitory ones for acute lymphoblastic leukemia, also absence of L5178Y lymphoblasts was determined. Curves A and B, formation of cinnamic acid in the medium in the presence and absence of cells, respectively. Curves C and D, formation of cinnamic acid in the medium in the presence and absence of cells, respectively. Curves E and F, formation of coumaric acid in the medium in the presence and absence of cells, respectively.

Phenylalanine ammonia-lyase (0.5 to 3.0 units), which were inhibitory ones for acute lymphoblastic leukemia, also prevented subsequent cell growth of murine lymphoblasts, but less rapidly than with higher concentrations of enzyme.

Because proliferating cells have a higher metabolic rate and thus a greater requirement for amino acids, it was of particular interest to find that growth of leukemic cells and PHA-stimulated normal cells was inhibited by phenylalanine ammonia-lyase whereas normal cells, when maintained in the resting state, were relatively resistant. Roberts and Simonsen (22) and Ryan and Lorincz (23) also found that concentrations of several amino acids in malignant tumors are from 1.3 to 11 times greater than in surrounding normal tissues. This finding suggests that other neoplasms in addition to leukemias require relatively high concentrations of amino acids for growth. Thus, the increased demands for amino acids of rapidly dividing malignant cells may provide an important basis for the effective treatment of neoplasia with enzymes.

Of further interest was the finding that greater than 98% of the L5178Y murine lymphoblastic cells were nonviable following treatment with phenylalanine ammonia-lyase for 3 days. When these cells were retreated with phenylalanine ammonia-lyase to twice the original enzyme concentration, however, the percentage of viable cells was not altered. Three possible explanations for these findings are: (a) a population of cells, which preexists or is selected, is resistant to deprivation of phenylalanine; (b) a small amount of phenylalanine remains in the medium, which is sufficient to maintain a small number of viable cells and; (c) the remaining lymphoblasts may have lost their capacity to divide.

The 1st possibility appears to be unlikely for 2 reasons. Phenylalanine cannot be synthesized by the mammalian cell. Also, the induction of a cellular enzyme which would inactivate phenylalanine ammonia-lyase does not occur, since phenylalanine ammonia-lyase activity was observed to be constant for at least 2 weeks in the presence of cells. Furthermore, although only 75% of the tyrosine present was removed by phenylalanine ammonia-lyase, this nonessential amino acid does not meet the phenylalanine requirement. The 2nd explanation appears to be feasible. Although it was demonstrated (Chart 4) that 100% of the phenylalanine in the medium was converted to cinnamic acid, this assay is only significant to within 3 to 4%. Thus, a small but undetectable amount of phenylalanine (<0.2 μM) may be present in the medium and may be sufficient to maintain a small population of cells. In support of this suggestion is the fact that the equilibrium constant (9) for the deamination reaction predicts at equilibrium that approximately 0.02% of the original phenylalanine (<0.08 μM) remains in the medium. The 3rd possibility is intriguing because this explanation implies that lymphoblasts that have been treated with phenylalanine ammonia-lyase are converted to a nondividing state. Additional studies in progress which explore the effects of different concentrations of phenylalanine ammonia-lyase on DNA synthesis should provide an increased understanding of the biological characteristics of these cells.

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