Therapeutic Properties of a New Glutaminase-Asparaginase Preparation and the Influence of the Lactate Dehydrogenase-elevating Virus

Vernon Riley, Darrel Spackman, M. A. Fitzmaurice, Joseph Roberts, John S. Holcenberg, and William C. Dolowy


SUMMARY

A new enzyme termed GA: 1.2, possessing approximately equal amounts of glutaminase and asparaginase activity, has antitumor activities against cancers other than leukemia, thus enlarging the potential for cancer therapy by amino acid deprivation. When tested against the asparagine-dependent EARED-1 leukemia in the presence of the lactate dehydrogenase-elevating virus (LDH-virus), striking tumor regression was obtained. This asparagine-dependent mouse tumor was used as a model to study the basic mechanism of the therapeutic action of the enzyme and its influence upon various plasma amino acids in the presence and absence of the LDH-virus. At doses of 150 IU/kg and higher, both plasma glutamine and asparagine were depleted to undetectable levels (less than 1 nmole/ml) when the virus was present but not in its absence. Tumor regression was correlated with asparagine and glutamine depletion in the plasma. Although there were no measurable differences in the plasma glutamine and asparagine depletion below 1 nmole/ml as a function of increasing enzyme dose, both glutamic and aspartic acids increased systematically with dose. The plasma half-life of GA: 1.2 is 1 to 2 hr in normal mice, but was increased to 12 to 18 hr when mice were infected with the LDH-virus. Thus, the presence of the LDH-virus in the host is necessary for expression of the therapeutic capabilities of the enzyme preparation on this and presumably other mouse tumor systems.

INTRODUCTION

A new enzyme, isolated by Roberts et al. (24–26) from a gram-negative soil bacillus, possesses the exciting capabilities of inducing tumor regressions in neoplasms other than the usual leukemias and lymphomas, the cancers that have been primarily susceptible to Escherichia coli asparaginase therapy. The discovery of this more comprehensively acting enzyme thus carries the promise of extending the basic concept inherent in asparaginase therapy to a much broader spectrum of cancer varieties (23).

The enzyme preparation has been designated GA: 1.2 by Roberts et al. This nomenclature carries the information that the enzyme possesses approximately equal amounts of glutaminase and asparaginase activity; actually, it has a ratio of 1.2 to 1.0 (26).

The physiological half-life of this enzyme in normal, healthy mice is only 1 to 2 hr. However, in mice infected intentionally or otherwise by the LDH-virus, the half-life is increased more than 10-fold, to give a T1/2 of from 12 to 18 hr. This ability of the LDH-virus to induce impaired clearance of administered GA: 1.2 is also effective for EC-2 asparaginase (13). The LDH-virus thus affects the clearance of both EC-2 asparaginase and GA: 1.2 glutaminase-asparaginase in a similar manner. This and other capabilities of the LDH-virus for altering host physiology become important factors in assessing the potential therapeutic properties of enzymes, whether it be EC-2 asparaginase or new enzymic preparations (13, 15, 17, 20–23, 28).

Because of the dual-enzyme activities of the GA: 1.2 preparation, it seemed desirable to study its basic properties in a tumor model that was known to be sensitive to at least one of the potentially therapeutic enzymic properties. The use of transplantable mouse leukemia-lymphoma EARED-1 appeared in advance to be appropriate, since this tumor had been used in our laboratories and elsewhere for EC-2 L-asparaginase studies. It was thus known to have asparagine dependencies, and its basic biological behavior was established in respect to growth and regression during asparaginase therapy in the presence and absence of the LDH-virus. Also of some importance in tumor biology is the ability to distinguish purely chemotherapeutic effects from supplementary immunological influences on tumor regres-

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2 To whom reprint requests should be addressed, at Department of Microbiology, Pacific Northwest Research Foundation, 1102 Columbia Street, Seattle, Wash. 98104.

3 Present address: Sloan-Kettering Institute, Rye, N. Y.

4 These abbreviations used are: LDH-virus, lactate dehydrogenase-elevating virus; RES, reticuloendothelial system.

sion. In this connection, the EARAD-1 tumor is synergistic with the mouse strain used; this genetic factor minimizes uncontrolled immunological effects on the tumor (11).

MATERIALS AND METHODS

Enzyme Isolation and Purification

Glutaminase-Asparaginase GA:1.2. GA:1.2 was purified by Roberts (26) from a soil isolate identified as Acinetobacter glutaminisicans (ATCC 27197). A homogeneous glutaminase-asparaginase preparation was obtained by means of cation- and anion-exchange column chromatography and ammonium sulfate fractionation (24–26).

Enzyme isolation and purification was conducted at 0–7°. Packed cells were suspended in 3 to 4 volumes of 0.05 M potassium phosphate buffer, pH 7.2, and sonically disrupted with a Branson 20-kc sonifier for 5 min. The sonic extract was centrifuged for 15 min at 2,000 × g. The sediment was resuspended in 3 to 4 volumes of buffer and subjected again to sonic disruption and centrifugation. The supernatants were combined and centrifuged at 9,000 × g for 45 min. The pH of the enzyme-containing cell extract was lowered to 6.5 by dropwise addition of phosphoric acid, and the nonsoluble constituents were removed by centrifugation at 9,000 × g for 30 min. Approximately 400 ml of the supernatant fluid, which contained about 9 g of protein and 16,000 IU of L-glutaminase activity, were applied to a CM-Sephadex column (5 x 80 cm) that had been equilibrated with 0.04 M sodium phosphate buffer, pH 6.5. The enzyme was eluted with 3 liters of a linear gradient of 0 to 1 M NaCl in 0.04 M sodium phosphate buffer, pH 6.5, at a flow rate of about 140 ml/hr. Ten-ml fractions were collected and assayed for L-glutaminase activity. The glutaminase-asparaginase fraction was eluted from the CM-Sephadex column at between 0.15 to 0.25 M NaCl concentration in a total volume of about one-third that of the starting extract.

The active fractions were combined and the pH of the pool was adjusted to 7.2 with sodium hydroxide. Solid ammonium sulfate (315 g/liter) was added slowly to the enzyme solution while a pH of 7.2 was maintained by dropwise addition of ammonium hydroxide. After 30 min at 4°, the precipitate was removed by centrifugation. Ammonium sulfate (255 g/liter) was then added to the supernatant and the resulting precipitate was collected by centrifugation, resuspended in a minimal volume of 0.01 M sodium phosphate buffer, pH 7.2, and dialyzed against the suspending buffer. The dialyzed enzyme solution, (60 ml) which contained about 100 mg of protein and 10,000 IU of L-glutaminase activity, was adjusted to pH 8.0 with dilute NaOH and applied to a DEAE-Sephadex column (2.5 x 35 cm) which had been equilibrated with 0.01 M sodium phosphate buffer, pH 8.0, at a flow rate of about 35 ml/hr. The glutaminase-asparaginase pool of fractions that appeared at the front was adjusted to pH 7.2 with dilute HCl, lyophilized, and stored at 5°.

The enzyme obtained from the above-described step was dissolved in cold water and dialyzed overnight against 1 mM sodium phosphate buffer at pH 6.5. The dialyzed material, containing 2 to 5 mg of protein per ml, was clarified by centrifugation. The enzyme solution was kept at 0°, and 1 volume of cold absolute ethanol was slowly added with mixing. A slight turbidity which at times appeared was immediately removed by centrifugation. Crystallization was allowed to proceed at 5° overnight. The crystals were collected by centrifugation and washed with ethanol: water (1:2:1, v/v) and lyophilized. The dried crystals were stable upon storage at 5°. Repeated crystallizations yielded a constant specific activity (180 ± 20 IU/mg of protein). The crystals were needle shaped, approximately 70 x 3.5 μm. Freezing and thawing of the highly purified glutaminase-asparaginase resulted in a substantial loss (40 to 50%) of activity. However, the lyophilized enzyme was stable during prolonged storage and for at least 10 days at 5° in solution.

Purity of GA:1.2 Glutaminase-Asparaginase. The antherone determination for carbohydrate and the esterified phosphorus determination for phospholipid were negative. Disc gel electrophoresis in sodium dodecyl sulfate yielded a single protein band.

Isoelectric Point. The isoelectric point of the GA:1.2 glutaminase-asparaginase determined by isoelectric focusing on ampholytes was 8.38 with the pH 3 to 10 carrier, and was 8.49 with the pH 7 to 10 carrier. The isoelectric point of the L-asparaginase activity in the glutaminase-asparaginase preparation was the same as the glutaminase activity.

Effect of Substrate Concentration and pH on Activity. The standard assay utilizing nesslerization is linear with enzyme concentration to at least 0.6 IU/reaction tube and 60 min with time. The initial enzyme reaction rates for the deamination of L-glutamine and L-asparagine at varying substrate concentrations were determined at pH 7.5. Double reciprocal Lineweaver-Burk plots of these data yield a straight line between 1.9 and 190 μM asparagine. The Km values were 4.8 x 10−⁶ M for asparagine and 5.8 x 10−⁶ M for glutamine. Citrate, acetate, phosphate, barbital, borate, or Tris ions did not appreciably affect enzyme activity. Both glutaminase and asparaginase activity was maintained over a pH range 6 to 9, with near optimal activity obtained at a physiological pH of 7.4.

Enzyme Specificity. The enzyme catalyzes the hydrolysis of both D and L isomers of glutamine and asparagine, but the activity toward the L isomer was about 3 times higher than that of the D isomer.

Glutaminase G-1. The G-1 glutaminase was purified by Holcenberg (6) from a soil-isolated gram-positive coccus belonging to the Sarcina genus. The bacteria were grown in a standard salt mixture containing 0.2% yeast extract, 0.5% ammonium sulfate, and 1% glucose. The organisms were disrupted sonically following treatment with 0.5% lysozyme and 0.2% Lubrol detergent. Protamine sulfate (10 mg/liter) was added to the sonic extract, and the mixture was centrifuged for 10 min at 30,000 × g. The supernatant was treated with increments of cold absolute ethanol.

The fraction precipitating between 9 and 34% ethanol was resuspended and chromatographed on a DEAE-cellulose column; a sodium chloride linear gradient was used, with 0.5 M Tris-HCl buffer, pH 7.5, containing 10 mM MgCl₂ and 10 mM 2-mercaptoethanol. The enzymically active fractions were absorbed on calcium phosphate gel at a ratio of...
Amino Acid Determinations

Amino acid assays were carried out with a Beckman Model 120B amino acid analyzer, modified to provide accelerated, semiautomatic runs. Recently revised and improved methods of analysis were used, with sodium citrate buffers for the basic amino acids and lithium citrate buffers for the acidic and neutral amino acids (1, 29, 31). The latter buffer system is essential for the satisfactory separation of asparagine, glutamine, and glutamic acid.

Experimental Groups

Mice used in a given experiment were from uniform groups in terms of age, sex, strain, and origin. Mice were received from reliable commercial breeders in batches of 50 or 100, with their age range limited to about 5 days. Upon receipt, the animals were randomly distributed into groups of 10 and were held in quarantine for a minimum of 2 weeks prior to use. Any abnormal-appearing mice, or those departing from our specifications of 19 to 22 g, were discarded. The establishment of mouse social order equilibration is of importance in maintaining animals under conditions of minimum stress in order to avoid adrenocortical reactions with their attending alterations in the thymus, T-cells, and immunological competence (4, 17, 28, 30). Thus, the segregated mouse groups used for equilibration and storage were not ordinarily altered or remixed prior to their use in experiments. Unless otherwise indicated, each experimental group consisted of 10 uniform mice.

Animal Environment

Standard plastic cages, 11.5 x 7 x 5 inches, containing 0.50 to 0.75 inch of San-I-Cel ground corncob bedding were used to house 5 to 10 mice. In our experience, plastic cages have proven to be superior to metal cages in terms of insulation, resistance to temperature changes, and minimization of sound and thermal stress factors. Inasmuch as endocrine and diurnal effects are related to light and dark exposures, the plastic cages are also preferable for controlling admittance of light to the animals. Standard 12-hr intervals of light and dark were controlled by automatic clock switches.

The mouse diet consisted of Wayne Lab Blox pellets supplied through the cage-top food hopper. This nutritionally complete diet was available to the mice at all times, except when fasting was intentionally imposed to control plasma amino acids or for other experimental reasons. Clean water was available at all times by way of pint glass bottles and stainless steel drinking tubes. High standards of sanitation were employed in the routine changing and sterilization of drinking bottles, tubes, and cages. Cage bottoms and bedding were changed once or twice a week, depending upon experimental and other circumstances.

All animals, including both stock and experimental groups were protected by a special barrier system consisting of ventilated enclosed shelves provided with filtered laminar flow air, vented outside of the building following contact with the mice and their generated aerosols (16).

Blood Sampling Techniques

Special sampling procedures were used to minimize amino acid alterations during the processing of the blood and plasma samples for amino acid analysis. Blood samples were removed just prior to the injection of asparaginase or glutaminase in those experiments in which multiple enzyme doses were given; thus, the blood samples reflect the amino
Acid concentrations at the time of minimal enzyme levels. Blood samples of about 0.2 ml/mouse were removed by the orbital bleeding technique (14), with the use of prechilled, disposable, sodium-heparinized bleeding tubes. The orbital bleeding procedure requires only 5 to 10 sec for the actual removal of the blood; the blood-filled tubes were quickly stoppered and plunged into an ice bath, chilling the blood immediately to 0°. The blood samples were centrifuged at 0°, and the cold plasmas were separated and transferred without delay to preweighed, disposable tubes containing the protein-precipitating agent, sulfosalicylic acid, which stopped all enzyme activity and stabilized the amino acid pattern.

Tumor

Syngeneic, asparaginase-sensitive, transplantable leukemia EARAD-1, used in these experiments, was originally obtained from Dr. L. J. Old of the Sloan-Kettering Institute (11). Tissue culture passage of the stock tumor was used to free the tumor of the LDH-virus, inasmuch as EARAD-1 leukemia cells do not support replication of this virus under in vitro conditions.

Tumor Implantation and Measurements. The EARAD-1 leukemia will grow as an ascites form when injected i.p., or will grow as a solid lymphomatous tumor when implanted i.m. or s.c. In order to follow the tumor growth and regression, we used the solid form, and the tumor mass was measured by calipers.

The donor tumors were harvested aseptically and injected into recipient mice as a 20 to 50% suspension of free cells and small fragments of lymphoma tissue in 0.9% NaCl solution. This suspension was prepared by forcing the tumor tissue through a 2.5-ml disposable syringe fitted with an 18-gauge needle, and into a sterile, rubber-stoppered bottle of appropriate size. The tumor suspension was forced in and out of the syringe and bottle unit until a suitable tissue suspension was obtained. This preparation was injected into the upper part of the hip, where 3-dimensional tumor measurements could be easily made with calipers. The injecting needle was passed through the musculature of the leg, and 0.1 ml of suspension was deposited s.c. at the upper hip-back junction.

The 3 caliper measurements were made at right angles to each other, as if measuring a cube. Although the tumors were not perfectly regular, a close approximation to true cubic volume was obtained by a slight compression of the tumor during the 3 measurements. As an example, the volume of a small rubber ball can be determined with this procedure by applying about 10% compression during each measurement. This technique yields good tumor volume reproducibility between experiments and between different technicians if their measuring procedures are consciously compared.

Material Sources

Lubrol WX (5070) detergent was obtained from ICI American, Stamford, Conn. Lysozyme was from Worthington Biochemical Corp., Freehold, N. J. Protamine sulfate and Tris buffer base were from Sigma Chemical Co., St. Louis, Mo.; DEAE-cellulose was from H. Reeve Angel and Co., Clifton, N. J.; DEAE-CM-Sephadex was from Pharmacia Fine Chemicals, Piscataway, N. J.: asparaginase and glutamine were from Calbiochem, La Jolla, Calif.; enzyme grade ammonium sulfate was from ICN Nutritional Biochemicals Div., Cleveland, Ohio; San-I-Cel laboratory animal bedding was from Paxton Processing Co., Inc., Paxton, Ill.; and Wayne Lab Blox mouse food pellets were from Northwest Pet Products, Milwaukie, Ore.

RESULTS

Influence of the LDH-Virus. The therapeutic effects of GA: 1.2, a combined glutaminase-asparaginase molecule, like those of EC-2 and other asparaginas, were first successfully demonstrated in mice bearing tumors that were contaminated with the LDH-virus. Infection of the host with this virus dramatically improves the effectiveness of asparaginase against asparagine-dependent tumors in vivo (15, 18, 20–22, 28). This enhancing effect is at least partially the result of a 5- to 8-fold impairment in EC-2 asparaginase clearance from the plasma of LDH-virus-infected mice (18, 20).

Clearance of the glutaminases associated with these preparations (EC-2 or GA:1.2) are also impaired in LDH-virus-infected mice.

The data shown in Chart 1 compare the effect of 2 dose levels of GA:1.2 against the asparaginase-sensitive EARAD-1 lymphoma in the presence and absence of LDH-virus infection. Both concentrations of GA:1.2 (150 and 300 IU/kg) were completely ineffective therapeutically in the absence of the LDH-virus infection. This is shown by the tumor growth curves, which are essentially the same as that of the untreated control mice. In contrast to the therapeutic ineffectiveness of GA:1.2 against this tumor in noninfected mice, the enzyme-treated, LDH-virus-infected mice exhibited tumor regression that continued for the duration of the enzyme treatment period. The LDH-virus by itself has no tangible effect upon this tumor growth, as shown by similar growth curves of virus-infected and noninfected tumor control groups. However, to minimize any covert influence on the tumors, the virus was not introduced until 3 days prior to the initiation of GA:1.2 treatment.

Correlation of Therapeutic Effects of GA:1.2 with Depletion of Plasma Asparagine and Glutamine. Chart 2 shows the tumor regression observed during treatment of EARAD-1 lymphomas with daily doses of 300 IU of GA:1.2 per kg in LDH-virus-infected mice, and the immediate regrowth of the tumors following cessation of the enzyme treatment. Closely paralleling this tumor growth is the reappearance of both asparagine and glutamine in the circulating plasma. These amino acids were rapidly removed from the plasma upon initiation of the GA:1.2 treatment and were maintained at levels of less than 1 nmole/ml of plasma in the virus-infected mice while the tumors were regressing.

Failure to observe therapeutic effects by GA:1.2 in the absence of the LDH-virus is correlated with the rapid return
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Chart 1. A comparison of the therapeutic effects of a glutaminase-asparaginase (GA: 1.2) preparation in the presence and absence of the LDH-virus. The virus was introduced 72 hr prior to the enzyme treatment. Daily GA: 1.2 administration was started on Day 14, and the last dose was given on Day 22; thus each experimental mouse received 9 i.p. injections of enzyme at the doses indicated. Tumor control mice, with and without the LDH-virus, received sham injections. Since the virus had no influence on tumor growth in the absence of enzyme, the tumor growth curve of the controls is represented by a single line. There were 9 mice in each experimental group and 18 tumor control animals.

Chart 2. Correlation of glutamine and asparagine depletion of the blood plasma during asparaginase-glutaminase (GA: 1.2) treatment, with regression of the established tumors. Reappearance of these 2 amino acids in the plasma was closely associated with the recurrent growth of the tumor. The LDH-virus had been introduced 3 days prior to therapy and was thus in its acute viral phase. GA: 1.2 administration was started on Day 14, and the last dose was given on Day 22; thus each experimental mouse received 9 i.p. injections of enzyme at the dose indicated. Tumor regrowth was detectable by Day 25, at which time both asparagine and glutamine had reappeared in the plasma.

Comparison of the Therapeutic Effectiveness of Glutaminase and Asparaginase against the EARAD-1 Leukemia. Chart 3 compares the therapeutic effects of EC-2 1-asparaginase, G-1 glutaminase, and GA:1.2 glutaminase-asparaginase against the asparagine-dependent EARAD-1 lymphoma. All the enzyme preparations were tested in mice intentionally infected with the LDH-virus. The maximum dose (1,000 IU/kg) of GA:1.2 was less effective therapeutically against this tumor than was 250 IU of EC-2 asparaginase per kg. This high dose of GA:1.2 also was toxic, with all of the mice in this group dying 3 days after the 5-day treatment period without attaining complete tumor regression. Tumor regression was observed in the group treated with a daily dose of 250 IU of EC-2 asparaginase per kg. In contrast to the tumor regressions obtained with GA:1.2 and EC-2 asparaginase, the enzyme G-1 was completely ineffective against the EARAD-1 lymphoma at a dose of 270 IU kg/day, even in LDH-virus-infected mice. Since G-1 has no direct effect upon the plasma asparagine, its effectiveness against the asparagine-dependent EARAD-1 lymphoma would not be theoretically expected unless the EARAD-1 tumor were also glutamine dependent. This possibility was not determined since plasma glutamine was not reduced to presumed therapeutic levels (Table 1). It is also possible that the high K_m of the G-1 glutaminase (5 x 10^-4 M) is responsible for its lack of effectiveness with this tumor, in contrast to the K_m of 5 x 10^-6 M possessed by the glutaminase of GA:1.2 (6). Thus the question of glutamine dependence for this tumor is still open.

Rechallenge of Recurring Glutaminase-treated Tumors with EC-2 Asparaginase. The G-1 glutaminase-treated mice were subsequently treated with 12 daily doses (50 IU/kg/day) of EC-2 asparaginase (Group A), beginning on the last day of G-1 treatment. The results are shown in Chart 3. The dramatic regression exhibited during the period of EC-2 asparaginase administration indicates that these tumors were still asparaginase-sensitive following treatment with G-1 glutaminase. Chart 4 shows a continuation of the tumor behavior of these doubly treated animals (Group A). In this group, tumor regrowth commenced immediately following cessation of the daily doses of EC-2 asparaginase.

Group B consists of the tumors recurring after 9 days of tumor remission, which were induced by 5 daily doses of EC-2 asparaginase at 250 IU/kg/day (Chart 3). Since the recurring tumor growth curves of these 2 groups were similar but the history of their treatment differed, they were both rechallenged with 1 large dose of EC-2 asparaginase (5,000 IU/kg) on Day 37 after the initial tumor implantation. Group A tumors were completely insensitive to this relatively large dose of EC-2 asparaginase, while Group B
Table 1
Comparison of action of GA:1.2 and G-1 on removal of plasma asparagine and glutamine
A single i.p. enzyme dose was given.

<table>
<thead>
<tr>
<th>Time postinjection</th>
<th>No virus GA:1.2 (300 IU/kg)</th>
<th>With virusa GA:1.2 (300 IU/kg)</th>
<th>With virusa G-1 (300 IU/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asn</td>
<td>Gln</td>
<td>Asn</td>
</tr>
<tr>
<td>0.5 hr</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 day</td>
<td>32</td>
<td>430</td>
<td>0</td>
</tr>
<tr>
<td>2 days</td>
<td>38</td>
<td>427</td>
<td>0</td>
</tr>
<tr>
<td>3 days</td>
<td>31</td>
<td>501</td>
<td>0</td>
</tr>
<tr>
<td>5 days</td>
<td>28</td>
<td>418</td>
<td>0</td>
</tr>
</tbody>
</table>

*a The LDH-virus was inoculated i.p. 3 days prior to initiation of treatment.

Chart 3. Comparison of various enzyme preparations and their therapeutic influences upon a lymphoma-leukemia known to be sensitive to EC-2 L-asparaginase (A-ase). G-1 is a purified glutaminase possessing no asparaginase activity, GA:1.2 possesses approximately equal glutaminase and asparaginase capabilities, and EC-2 is predominately an asparaginase having less than 5% glutaminase activity. The LDH-virus was introduced 3 days prior to the initial treatment period. Enzyme administration was initiated on Day 14 and continued daily through Day 18, except in the case of Curve A, where EC-2 asparaginase was administered for 12 days to mice that had received previous injections of G-1 glutaminase. There were 10 BAF female mice in each experimental group and 20 tumor control mice.

Tumors exhibited a moderate degree of asparaginase sensitivity. Since both the asparagine dependency of the recurring tumor cells and the host immunological response to the administered enzymes may be involved, these results are difficult to interpret without further testing.

LDH-Virus Influence on GA:1.2 Clearance. Chart 5 illustrates the striking difference observed in the host clearance of the injected GA:1.2 enzyme preparation between normal and LDH-virus-infected mice. A normal mouse eliminates this preparation rapidly, with a t_1/2 of 1 to 2 hr. In contrast, mice that have received injections of LDH-virus exhibit a relatively slow rate of removal for this exogenous enzyme, yielding a t_1/2 of 12 to 18 hr. Mice that have received implants of experimental tumors do not show any striking alterations in their clearance ability, compared with normal animals (13), unless the tumor is infected with the LDH-virus, in which case the disappearance of the enzyme from the plasma is substantially retarded, giving again a t_1/2 of 12 to 18 hr. Although the tumor itself may eventually influence both clearance and enzyme influx in the host, the primary factor responsible for the physiological changes observed here is the presence of the LDH-virus (19). These relationships are partially illustrated in Chart 5.

It is tempting to conclude that the improved therapeutic effect of GA:1.2 upon tumor regression seen in the presence of the LDH-virus is totally due to the virus-induced impaired clearance of the enzyme. Although this is probably an important factor in the therapeutic efficacy of GA:1.2, other studies with this tumor system, but with the use of...
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EC-2 L-asparaginase, have demonstrated that there are additional virus-associated factors that may have to be considered. Examples include the influence of the virus upon the immunological capacity of the host and possibly other concomitant biochemical alterations that it induces (3, 4, 13, 15, 16, 18, 20–23, 28, 30).

Toxicity of GA: 1.2 Enzyme. Although the GA: 1.2 enzyme exhibited therapeutic effects against the EARAD-1 lymphoma, as indicated by regression of established tumors, the treated animals eventually all died. Since this tumor has developed a resistant population of cells to asparaginase during repeated passages, some of the mice treated with EC-2 asparaginase also eventually experienced tumor recurrence and died with large asparaginase-resistant tumors (Chart 4, Group A). However, as already indicated in Chart 3, animals that received GA: 1.2, in a dose of 1,000 IU/kg/day for 5 days, died before their recurring tumors attained a volume that would ordinarily be expected to kill the mice. Although GA: 1.2 administration caused tumor regression and prolonged survival (Table 2), the treated mice died with much smaller tumors than did either the controls or the EC-2 asparaginase-treated mice that died with recurrent tumors. Some mice treated with GA: 1.2 died with large recurring tumors, as indicated in Chart 2. However, the mice receiving GA: 1.2, at 1000 IU/kg/day for 5 days, died with tumors averaging only about 1.0 cu cm in volume. The large quantities of glutamate generated in the plasma of mice treated with this enzyme may explain these latent toxic effects (20, 21).

In an experiment designed to explore the possible toxicity of high concentrations of plasma aspartate and glutamate in mice, solutions of the sodium salts of aspartic and glutamic acids at pH 7.2 were administered as single dose injections, both i.v. by the tail vein and i.p. None of the injections, which included doses up to 300,000 nmoles, caused any deaths or overt toxicities. Levels of glutamate in excess of 5,000 nmoles/ml were found when the plasma was tested within 5 min following i.p. injections; however, by 1 hr following the i.v. injections, the glutamate and aspartate levels were nearly normal, indicating a rapid clearance or biochemical conversion. Thus, additional testing is needed to quantitatively define the potential toxic capabilities of these metabolites associated with asparaginase and glutaminase therapy.

**DISCUSSION**

The Half-Life of Therapeutic Substances. The total absence of detectable therapeutic effect with the use of GA: 1.2 in this tumor system in the absence of the LDH-virus raises questions of far-reaching significance to chemotherapy. It is clear from the data that the primary explanation for failure to demonstrate therapy is not due to the intrinsic potentials of the enzyme itself, but to its inadequate half-life in the mouse, a condition that is remedied by the presence of the LDH-virus. This fact implies that there may be many potentially valuable therapeutic substances whose efficacy was unappreciated or missed because of rapid clearance or other circumstances that do not permit the drug to act effectively. For example, had this potentially valuable enzyme been first tested against tumors that were not accidentally contaminated with the LDH-virus, its therapeutic properties would have appeared falsely negative.
...and a valuable compound would have been discarded. It would thus seem desirable to determine the $t_{1/2}$ of all preparations that are being tested for therapeutic properties, inasmuch as rapid elimination may automatically preclude their usefulness unless means can be found to appropriately extend their half-life.

These experimental observations also reemphasize the potential value of the LDH-virus in such test systems. Since the virus is essentially benign and persists for the life of the host, it can be used consciously, rather than by accident, in mouse systems that are being utilized for the testing of a wide variety of drugs, the clearance of which may be beneficially slowed by the virus. The practical utility of this has been demonstrated by the significant enhancement of asparaginase therapy, as previously reported (15, 16, 20–23, 30).

**LDH-Virus Impairment of Antibody Formation.** The striking alterations in GA:1.2 clearance time ($t_{1/2}$) of mice infected with the LDH-virus suggest that this phenomenon is a satisfactory explanation for the dramatic difference in the therapeutic capacity of the GA:1.2 enzyme preparation. Charts 1 to 3 reveal that the quantitative differences in tumor regressions, when the enzyme varied between 150 and 1,000 IU/kg/day, were not strictly proportional to the dose. In the case of previous EC-2 asparaginase therapy, when the dose of this enzyme was increased in the absence of the LDH-virus to equal asparaginase blood levels comparable to the concentrations present during virus infection, the therapeutic effects were still not as good as when the virus was present (20). It was these observations that led to a further examination of the influence of the LDH-virus. It has been found, in the case of EC-2 asparaginase, that the LDH-virus also impaired the early appearance of antibodies directed against the therapeutic enzyme (21, 30). It is therefore prudent to consider the possible importance of these additional virus-induced effects in evaluating its overall influence upon the therapeutic efficaciousness of GA:1.2.

One of the puzzling questions raised in examining the therapeutic capacity of GA:1.2, in comparison to EC-2 asparaginase, is that the latter asparaginase appears to exert a more striking therapeutic effect on this tumor than the same amount of asparaginase activity in the GA:1.2 preparation. As an example, compare the better tumor regression obtained with 50 IU/kg/day of EC-2 asparaginase (Chart 3) with the effect shown in Chart 1 using GA:1.2 at 150 and 300 IU/kg/day, which had asparaginase activity of 125 and 250 IU/kg/day.

This question becomes even more interesting in view of the fact that the asparaginase $K_m$ of GA:1.2 is more favorable ($5 \times 10^{-4}$) than the $K_m$ of EC-2 asparaginase, which is $1.5 \times 10^{-3}$ (2, 26).

**Toxicity of GA:1.2 Glutaminase-Asparaginase.** The increased toxicity associated with GA:1.2 provides an opportunity for experimental exploration of this important clinical question. It has been established in our laboratory that the glutamic acid production associated with GA:1.2 is substantially higher than that produced by EC-2 asparaginase (V. Riley and D. Spackman, manuscript in preparation). Since glutamic acid is neutralized in vivo to become ionized glutamate with known toxic potentials, such as those reported for monosodium glutamate, it is possible that this product may be responsible for some or most of the mouse toxicities observed (20, 21). As an alternate or additional possibility, toxic effects may also result from depletion of glutamine in the circulating plasma, and thus possibly in vital tissues. Such deprivation may induce cellular injury by essential amino acid depletion rather than by a toxicity resulting exclusively from the accumulation of the metabolic products of GA:1.2 therapy or from endotoxins associated with the enzyme.

**The Problem of Plasma Glutamine Depletion.** The lack of response of the EARAD-1 leukemia-lymphoma to the G-1 enzyme preparation, which exhibits only glutaminase activity, implies that this cancer has a low or possibly no exogenous glutamine dependency. However, it would be premature to accept this conclusion without reservations, inasmuch as the G-1 preparation was not capable of depleting glutamine to less than 1 nmole/ml at an enzyme dose of 300 IU/kg/day, even in the presence of the LDH-virus (Table 1). In contrast, GA:1.2 at the same dose reduced both plasma asparagine and glutamine to "zero" within 30 min while, at the same time period but using G-1, the plasma glutamine concentration was reduced to only 40 nmols/ml. Twenty-three hr later, plasma glutamine in this group was back to 400 nmols/ml, and high glutamine levels continued throughout the period of daily enzyme administration, with concentrations fluctuating between 200 and 400 nmols per ml of plasma (Table 1). In all experimental groups, plasma samples were drawn just prior to the daily enzyme injections; thus, except for the 0.5-hr samples, the amino acid values cited indicate the levels found at approximately 23 hr post-enzyme injection, which would be the period of the lowest enzyme level, and thus of the highest amino acid concentration between injections.

Therefore, the question of the possible glutamine dependency of this tumor was not adequately tested, since this particular enzyme preparation, at the doses used, was not able to deplete and maintain glutamine at presumed therapeutic levels. This observation again points up the imperative need to monitor specific amino acids or other appropriate substrates in the plasma, on a time-course basis following the administration of enzymic or other therapeutic preparations, in order to know whether they are accomplishing the deprivations required to produce therapeutic conditions. It is necessary, of course, to also consider the high $K_m$ of the G-1 preparation ($5 \times 10^{-4}$ m) in evaluating its potential therapeutic properties.

**Enzyme Memory Effect?** An interesting question arises concerning a possible silent G-1 glutaminase effect upon the EARAD-1 tumor. Why did the recurring tumors, briefly treated with G-1 followed by EC-2 asparaginase treatment, exhibit resistance to rechallenge by EC-2 asparaginase? This nonresponsive behavior was in contrast to recurring tumors of similar size that had been treated with EC-2 asparaginase, which produced a primary tumor regression. Such tumors, when they recurred for the 2nd time, were again responsive to challenge by asparaginase in contrast to the recurring tumors that had an earlier exposure to the therapeutically ineffective G-1 preparation (Charts 3 and 4).
**LDH-Virus Effects.** These and related studies (2, 13, 15, 20, 21, 22) establish the vital importance of the clearance rate ($t_{1/2}$) in obtaining optimum therapeutic effects with anticancer enzyme preparations. Although the LDH-virus provides a useful means for favorably altering the $t_{1/2}$ of GA: 1.2, EC-2 asparaginase, and other materials in experimental models, it would obviously be desirable to accomplish similar effects for the benefit of patients. Three theoretical possibilities can be examined experimentally: (a) biochemical modification of the therapeutic enzyme to slow its removal rate from the tumor-bearing host without altering its enzyme activity, (b) biochemical simulation of the benign and useful effects of the LDH-virus in slowing the clearance mechanisms of the patient. Accomplishment of either or both of these possibilities would extend the usefulness of enzyme and other therapeutic preparations in clinical application; (c) a 3rd and possibly the most interesting influence of the LDH-virus is its ability to modify the normal immunological competence of the host. Although this has broad implications for oncogenic and other experimental models, its special interest in enzyme therapy is its suppression of antibody development against the administered enzyme proteins (21, 30). Normal immunological interference has an adverse effect upon the therapeutic potential of the enzyme as well as generating anaphylactic and other undesirable immunological responses in experimental animals and in cancer patients.

**Biochemical Possibilities for Simulating LDH-Virus Functions.** Studies that have either a direct or indirect bearing upon these theoretical possibilities for simulating the action of the LDH-virus include biochemical alterations of enzymes that extend their half-life in vivo (5, 27, 32), modifications of the RES or other physiological systems, which control the rate of clearance of the proteins (7, 8, 10, 12), and selective inhibition of the immunological apparatus, which produces interfering antibodies against administered therapeutic enzymes. Mahy and Rowson (personal communication) have significantly increased the $t_{1/2}$ of lactate dehydrogenase by acetylation of the lysine groups, without loss of enzyme activity. Hare and Handschumacher (5) have accomplished similar increases in the half-life of asparaginase by modifications with maleic anhydride, ethyl acetimidate, or methyl $\beta$-dimethylaminopropionimidate. Work is underway to encase asparaginase and other enzymes in micro-capsules which may greatly extend the enzyme half-life and reduce toxicities (W. C. Dolowy, personal communication). Of even greater potential benefit, such encapsulation may prevent or diminish the formation of neutralizing antibodies against asparaginase, glutaminase, and other therapeutic enzymes that may become available.

Wagner *et al.* (32) modified l-asparaginase by reacting it with nitrous acid to partially deaminate the enzyme. This alteration of the molecule extended its half-life without loss of enzymic activity. Rutter and Wade (27) correlated the half-life of a series of modified *Erwinia carotovora* asparaginase with their isoelectric points and found an optimum $t_{1/2}$ at a $pI$ of about 6, with a decrease in half-life on either side of this isoelectric $pH$. Mashburn and Landin (8) also associated differences in various asparaginases with small differences in isoelectric points. These and other studies offer promise that at least one of the beneficial effects produced by the LDH-virus may be simulated by directed manipulation of the enzyme molecule to increase its biological half-life.

Alternate or additional factors that may simulate the biological action of the LDH-virus, and thus improve enzyme therapy, involve modification or blockage of the host RES. The primary purpose of this would also be to slow the clearance capacity of the experimental animal or patient, in order to increase the half-life of administered therapeutic substances. Pearsall and Weiser (21) have reviewed the various factors that are known to influence phagocytosis and the RES, which are involved with physiological clearance. The persistence of foreign material in the blood plasma is partially determined by the physiological condition of the host, as well as by the properties of the materials, as indicated above. The rate of clearance of proteins that are removed by the RES macrophages is also influenced by the molecular weight and the aggregation state of the material, which may lead to 10-fold differences in the half-life (12, 27).

Since a permanent increase in the plasma levels of certain endogenous enzymes, as well as an impaired enzyme clearance capacity, are characteristic of mice infected with the LDH-virus, it has been speculated by Mahy *et al.* (7), Notkins (10), and others (4, 16, 19, 28) that these phenomena may be related to an impaired function of the RES. It has been suggested that this injury could result either from a specific action of the virus on the RES cells or a nonspecific effect due to blocking of the RES by prolonged phagocytosis of the persisting virus particles. More definitive studies are still required to establish the details of these intriguing physiological mechanisms.

Although immunosuppression is widely practiced both experimentally and with patients receiving kidney and other organ transplants, these techniques carry a special risk when used with cancer-bearing subjects, since immunosuppression may encourage the growth or metastases of the malignant cells. It appears, however, that the LDH-virus-induced suppression of antibodies against asparaginase has a beneficial rather than harmful effect upon the cancer therapy (15, 20, 21). Thus, further exploration of this virus-associated interference with the immunological apparatus may provide clues for selective immunosuppression.

**Malignant Cell Dependency.** One of the important consequences emerging from the development and testing of new enzymes for their antitumor properties is the opportunity to determine whether there is a broad category of amino acid dependencies among a variety of tumor types. It is thus appropriate to establish which amino acids may be more essential to tumor cells than to normal tissues, especially with respect to dependency upon the circulating plasma amino acid pool. When such dependencies upon specific amino acids can be established for specific tumors, a basis is provided for attacking such cancers through deprivation therapy.

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Therapeutic Properties of a New Glutaminase-Asparaginase Preparation and the Influence of the Lactate Dehydrogenase-Elevating Virus

Vernon Riley, Darrel Spackman, M. A. Fitzmaurice, et al.


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