Nitrogen Utilization in Mice Bearing Ehrlich Ascites Tumor Treated with \textit{Acinetobacter} Glutaminase-Asparaginase$^1$

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ABSTRACT

The effects of \textit{Acinetobacter} glutaminase-asparaginase (AGA) on protein and energy requirements were evaluated in mice bearing Ehrlich ascites tumors. In an initial experiment with normal mice, a zero protein diet resulted in a significant decrease in carcass nitrogen, liver nitrogen, and carcass energy relative to the animals on a normal, low, or high protein diet. In a second experiment, mice bearing Ehrlich ascites tumors were randomized into diet groups (zero or normal protein) and treatment groups (daily injections of AGA or 0.9% NaCl solution). In both treatment groups, the zero protein diet resulted in significant decreases in weight, liver nitrogen, carcass nitrogen, and carcass energy. Neither tumor nor AGA treatment affected body composition or the efficiency of nitrogen utilization. By Day 8, either the zero protein diet or AGA treatment significantly reduced ascites volume and tumor nitrogen content relative to controls. In a modification of Experiment 2, AGA treatment was stopped on Day 8, and all animals were given a normal protein diet. AGA, but not the zero protein diet, significantly enhanced ultimate survival. These experiments indicate that the requirements and utilization of energy and nitrogen are normal in mice with Ehrlich ascites tumor whether or not they are treated with AGA.

INTRODUCTION

Succinylated AGA$^3$ has antitumor activity in Phase I studies of children with acute leukemia (4, 8). The dose-limiting toxicities of this enzyme are central nervous system depression, nausea, weight loss, hypoproteinemia, and hyperglycemia with low insulin levels (4, 8). Some of those toxic problems could be caused by protein energy malnutrition due to decreased food intake during therapy or to defective protein utilization as a consequence of extracellular depletion of glutamine and asparagine. In a companion study (7), we showed that, during treatment of children with acute lymphocytic leukemia with this enzyme, ad libitum energy intake was below normal and that nitrogen balance was negative. Nevertheless, nitrogen balanced improved with amino acid supplementation, indicating that nitrogen utilization was normal in these children during treatment. The negative nitrogen balance and increased nitrogen requirement were largely a consequence of excessive urinary nitrogen losses. Increased nitrogen intake could improve the nutritional status of these children. In that small series, the amino acid supplementation did not prevent the antileukemia activity of the enzyme or increase its toxicity. Further studies are needed to determine whether improved nutritional state can decrease the toxicity of AGA.

COMPANION experiments were initiated to evaluate the effects of this enzyme on protein requirements and efficiency of nitrogen utilization in normal mice and mice bearing Ehrlich ascites tumors. These studies show that exogenous nitrogen utilization is normal in AGA-treated animals and that the antitumor effect of AGA is not altered by nutritional status. These results are consistent with those of the clinical study. In contrast with the clinical study, the mice did not have increased nitrogen requirements during AGA treatment.

MATERIALS AND METHODS

Animal Care. Female ICRF mice weighing 18 to 23.5 g were obtained from Sprague-Dawley, Madison, Wis. Four to 5 mice were housed in each plastic filter-top cage. A wire mesh was placed on the floor of each cage to minimize coprophagy. Animals were fed a normal protein diet (Table 1) for 4 to 7 days prior to the beginning of each of the 3 experiments. Water was provided ad libitum.

Experiment 1: Effect of Diet on Body Composition of Normal Mice. On Day 0, mice were randomized into 4 dietary groups (Table 1) (ICN Nutritional Biochemicals, Cleveland, Ohio). Four animals were killed on Day 0 for control values of body weight, liver nitrogen, carcass nitrogen, and carcass energy content. Food eaten by the animals in each diet group was weighed daily. Body weight was measured at least every other day. On Day 8, 4 animals on each experimental diet were killed, and body composition measurements were made (Table 2). Two additional mice from each group were killed, and their tissues were processed for amino acid analysis as described previously (5).

Experiment 2: Effect of Diet, AGA, and Tumor on Body Composition. Three days prior to the experiment, 0.1 ml of a 1:10 dilution of pooled plasma from mice infected with lactate dehydrogenase-elevating virus was injected i.p. into all mice in order to prolong the half-life of AGA (5). On Day 0, 4 animals were killed for control measurements of liver nitrogen, carcass nitrogen, and carcass energy. Twenty-four additional mice were randomized to receive one of 2 experimental diets, normal protein (I) or zero protein (II). These diets were used since they produced the greatest differences in body composition in the first experiment. All mice were inoculated with $10^6$ Ehrlich ascites tumor cells on Day 0. On Days 1 through 7, one half of the mice in each diet group received daily i.p. injections of...
AGA (300 IU/kg), and the other half received 0.9% NaCl solution. Body weight and food intake were measured daily. All mice were killed on Day 8. Body composition was analyzed on 4 animals in each group. Packed tumor cell volume in the ascites fluid and free tissue amino acid levels were determined on 2 animals in each group.

Experiment 3: Effect of Diet and AGA on Survival. Three days prior to the experiment, 32 mice received lactate dehydrogenase-elevating virus as in Experiment 2. On Day 0, mice were randomly assigned to receive either normal or zero protein diets and inoculated i.p. with 10⁶ Ehrlich ascites cells. On Days 1 to 7, 16 mice were treated with i.p. injections of AGA (300 IU/kg), and 16 received 0.9% NaCl solution. On Day 8, all mice were placed on a normal protein diet. Animals were weighed and examined for ascites daily until death.

Tumor, Liver, and Carcass Analysis. Animals were killed by fracturing the cervical spine. In animals bearing tumors, the abdominal fluid was aspirated, and the abdominal cavity was washed once with 2 ml 0.9% NaCl solution. The ascitic fluid and the washings were combined. In 4 animals in each tumor-bearing group of Experiment 2, the fluid was frozen for total nitrogen analysis by micro-Kjeldahl digestion followed by colorimetric determination of ammonia concentration (10). The total nitrogen content was analyzed on the liver and carcass of 4 mice from each group. The liver was removed and frozen. Prior to analysis, the liver was weighed, thawed and homogenized by hand in 10 ml water with a Potter-Elvehjem tissue grinder. The entire homogenate was assayed.

The intestines and stomach were removed from each mouse, and gastrointestinal contents were washed with a stream of normal 0.9% NaCl solution. The gastrointestinal tract was then returned to the carcass. The carcass (including stomach and intestines, but excluding liver) was frozen at −20°C in a plastic container. Prior to analysis, the carcass was weighed, softened by autoclaving for 15 min, and homogenized in a Sorvall Omnimixer with 100 ml of water at top speed for 3 min. Aliquots of the homogenate were used for total nitrogen analysis. Sixty-mll aliquots of this homogenate were also lyophilized. The dry weight of the lyophilizate was obtained, and pellets were fabricated for bomb calorimetry (Pellet Press, Parr Instrument Co., Moline, Ill.). The lyophilize was noted to be grossly homogeneous with no layering of particulate matter such as hair. These pellets were weighed and stored in a dessicator prior to bomb calorimetry using a Parr Adiabatic automatic bomb calorimeter (Parr Instrument Co.) (6, 11).

Liver, small intestine, brain, skeletal muscle, kidney, ascites fluid, and ascites cells were homogenized in cold sulfosalicylic acid and prepared for amino acid analysis as described previously (5).

Calculations and Statistical Analysis. There were 3 treatment groups in Experiment 2: no tumor; tumor; and tumor plus enzyme. For each of these treatment groups, NPU was calculated as described previously (1): NPU = [average carcass nitrogen on normal protein diet per initial body weight] − (average carcass nitrogen on zero protein diet per initial body weight)/nitrogen intake (g). The average carcass nitrogen (per initial body weight) was calculated for each of the treatment and diet groups. Nitrogen intake for each group was obtained from the average food intake (g) per animal in each group and from the manufacturer's estimate of nitrogen content (Table 1).

Statistical analysis was accomplished using 1-way and 2-way analysis of variance (2), 2-sample t test (2), Sheffe's test (2), and Dunnett's multiple comparison procedure (3).

RESULTS

Table 1
Experimental diet composition

<table>
<thead>
<tr>
<th>Diet component</th>
<th>Normal protein (I)</th>
<th>Low protein (II)</th>
<th>High protein (IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>27%</td>
<td>0%</td>
<td>8%</td>
</tr>
<tr>
<td>Digestible carbohydrate</td>
<td>59%</td>
<td>70%</td>
<td>78%</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>4%</td>
<td>4%</td>
<td>4%</td>
</tr>
</tbody>
</table>

*ICN Nutritional Biochemicals metabolic test diets: protein-free; low (8%) protein; normal protein, rat or mouse; high protein.

Table 2
Experimental diet on body composition of normal mice

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Normal (I)</th>
<th>Zero (II)</th>
<th>Low (III)</th>
<th>High (IV)</th>
<th>Control (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body wt (g)*</td>
<td>26.0 ± 1.0</td>
<td>14.1 ± 0.8</td>
<td>24.6 ± 2.3</td>
<td>25.3 ± 2.3</td>
<td>22.2 ± 2.0</td>
</tr>
<tr>
<td>Liver nitrogen (mg/g mouse)*</td>
<td>2.2 ± 0.17</td>
<td>0.8 ± 0.0</td>
<td>1.8 ± 0.17</td>
<td>2.3 ± 0.47</td>
<td>2.2 ± 0.19</td>
</tr>
<tr>
<td>Carcass nitrogen (mg/g mouse)*</td>
<td>25.7 ± 3.5</td>
<td>18.1 ± 1.1</td>
<td>24.9 ± 1.4</td>
<td>26.0 ± 3.2</td>
<td>23.7 ± 0.4</td>
</tr>
<tr>
<td>Carcass energy (kcal/g mouse)*</td>
<td>3.5 ± 0.51</td>
<td>1.0 ± 0.12</td>
<td>2.7 ± 0.37</td>
<td>3.4 ± 0.54</td>
<td>2.2 ± 0.19</td>
</tr>
</tbody>
</table>

*Results of liver nitrogen, carcass nitrogen, and carcass energy in each animal on Day 8 were expressed per gram body weight on Day 0 to normalize values for any differential changes in body weight during experimental diet periods.

The following pairs were significantly different from each other using the Sheffe's test (2): I versus II (p < 0.01); II versus IV (p < 0.01); II versus V (p < 0.01).

The following pairs were significantly different from each other using the Sheffe's test (2): I versus II (p < 0.01); II versus III (p < 0.01); II versus IV (p < 0.01); II versus V (p < 0.05); IV versus V (p < 0.05).
Normal Mice. Table 2 demonstrates that the zero protein diet had marked effects on weight gain and body composition, but there were not major differences among the other 3 diet groups. The zero protein diet resulted in significant decreases in liver nitrogen content and carcass energy content relative to controls; the mean carcass nitrogen content also decreased, but the \( p \) value was slightly greater than 0.05. Liver nitrogen, carcass nitrogen, and carcass energy in the animals on the zero protein diet were decreased relative to the values of animals on either the normal or high protein diet.

The average food intakes in the various groups were (g diet per g average initial mouse weight): 1.5 (I, normal); 1.2 (II, zero); 2.1 (III, low); and 1.2 (IV, high). The mice fed the zero protein diet ate a similar amount of food as did the normal protein group for 3 days and then had a steady decline in food intake. The high protein group ate less than did the normal group throughout the study.

Experiment 2: Effect of Diet, AGA, and Tumor on Body Composition. All animals survived during the 8-day experiment without gross changes except for weight loss in the zero protein groups and ascites in the tumor-bearing groups. Table 3 presents the results of this experiment. Control (Day 0) mice infected with lactate dehydrogenase-elevating virus had the same body composition as did the uninfected control mice in Experiment 1. Therefore, the data from Experiment 1 are presented (Group A) in this table to show the effects of diet on mice without tumor. Mice in Groups B and C bore Ehrlich ascites tumors; mice in Group C were treated with AGA. Table 3 shows that, in all groups, body weight, liver nitrogen, carcass nitrogen, and carcass energy decreased significantly in the mice fed a zero protein diet compared to those fed a normal protein diet. The animals that received AGA did not show significant differences in weight change relative to normal animals.

The changes in liver or carcass nitrogen or carcass energy were independent of the treatment group (A, B, or C). Based on the magnitude of the dietary effect in the 3 treatment groups, the efficiency of exogenous energy utilization or nitrogen utilization (liver or carcass analysis) was not significantly different in the 3 treatment groups (3). NPU, based on the carcass nitrogen content and the estimated food intake, was 0.12, 0.14, and 0.15, respectively, in Groups A, B, and C.

In mice treated with 0.9% NaCl solution, the weights of the packed ascites cells were 6% and 2% of the final body weight of the normal and zero protein diet groups. The total volumes of ascites fluid and cells were twice the packed cell volumes. Ascites may account for the slight increase in body weight in Group B compared to Group A or Group C. The nitrogen content of the cells and ascites fluid was 8% and 3% of the total body nitrogen of Groups Bl and BII, respectively. Treatment with AGA reduced the weight of the ascites cells and the volume and nitrogen content of the ascites fluid to near zero.

Mean food intake for the 8 days was between 1.3 and 1.6 g/g initial body weight for all groups of mice except those treated with AGA and zero protein diet (Group CII) in which it averaged only 0.6 g/g initial body weight. This marked decrease in energy and nitrogen intake was not reflected in a similar decrease in liver nitrogen, carcass nitrogen, or carcass energy.

Analysis of free tissue levels of aspartate, threonine, serine, asparagine, glutamate, glutamine, glycine, and alanine showed
that the zero protein diet had no general effect. The threonine levels in Ehrlich ascites cells, liver, muscle, plasma, small intestine, kidney, and brain were decreased in the zero protein group by 40 to 66%. The liver aspartate levels were 50%, and the liver glutamine levels were 160% of the normal protein diet group. Serine and glycine levels were elevated in the Ehrlich cells in the zero protein diet group (365 and 195% of normal protein group). Asparagine and glutamine levels were not decreased in Ehrlich ascites cells by the zero protein diet. Other values were within 25% of those from the normal diet group.

Treatment with AGA decreased the level of asparagine and glutamine in ascites fluid to below the level of detection (<0.003 mm). The tissue levels of glutamine decreased in muscle and increased in brain and kidney as described previously (5). Unlike that previous study, liver glutamine increased with AGA treatment in mice fed either diet. The zero protein diet + AGA group had a greater than 50% decrease in asparagine levels in brain and kidney while the normal protein + AGA group showed no change. Liver glutamate increased 4-fold in the zero protein diet + AGA group.

**Experiment 3: Effect of Diet and AGA on Survival.** The weight changes during the first 8 days of this study were very similar to those in Experiment 2 (Table 4). On Day 8, the AGA treatment was stopped, and all animals were fed a normal protein diet. Thereafter, all animals gained weight. Between Days 8 and 12, the zero protein groups treated with 0.9% NaCl solution and AGA gained an average of 5.5 and 9.3 g, respectively.

In this experiment and Experiment 2, either AGA treatment or a zero protein diet resulted in a significant decrease in ascites by Day 8. Nevertheless, all mice died of tumor (Table 4). AGA treatment produced a significant increase in survival. The zero protein diet did not prevent or enhance the antitumor effect of AGA. Furthermore, the zero protein diet had no effect on survival time in the control group treated with 0.9% NaCl solution.

**DISCUSSION**

Neither the presence of Ehrlich ascites tumor nor its treatment with AGA increased nitrogen or energy requirements in the study. Utilizing data relating to carcass nitrogen, liver nitrogen, and carcass energy content in animals ingesting either a normal protein or zero protein diet, we found that tumor or tumor + AGA treatment did not change nitrogen or energy utilization relative to normal mice. The validity of these conclusions is supported by our data showing that food (energy) intake in tumor-bearing animals did not exceed that in normal animals.

These results confirm our previous nitrogen balance studies in children with acute lymphocytic leukemia, which suggested that the efficiency of exogenous nitrogen utilization was not affected by AGA therapy (7). The children did have an increased nitrogen requirement due to large obligatory urinary nitrogen losses. The patients had a very low energy intake while the mice fed the normal protein diet had an adequate energy intake. Thus, the increased nitrogen requirement in the children may have been due to the effects of a low energy intake (9). However, species differences or individual characteristics of the 2 disease states, acute lymphocytic leukemia and Ehrlich ascites tumor, may also account for the different observations in the 2 studies.

Treatment with AGA for 7 days was associated with a marked decrease in ascites at the end of the course and a prolongation of ultimate survival. Mice fed a zero protein diet lost weight and had less ascites on Day 8 than did mice fed a normal diet. When the diet was changed to one containing normal protein, the mice rapidly gained weight, developed ascites, and died at the same time as did those fed a normal protein diet throughout the experiment. Furthermore, a zero protein diet did not enhance the antitumor effect of AGA.

These results differ from those of Ryan and Sornson (12) who reported synergism in the antitumor activities of a protein-free diet and asparaginase in mice bearing an asparagine-sensitive lymphosarcoma, 6C3HED. This difference is explained by the observation that a zero protein diet lowered the asparagine levels in the 6C3HED tumor but did not lower either asparagine or glutamine levels in the Ehrlich ascites tumor. If the asparagine levels of other tissues were not lowered to the same extent as the 6C3HED tumor by the zero protein diet, asparaginase might be expected to have a selective effect on the tumor. The zero protein diet did not produce a differential decrease in asparagine or glutamine levels of Ehrlich ascites tumor relative to normal mouse tissues and did not enhance the antitumor action of AGA.

In conclusion, our studies indicate that utilization and requirements for dietary nitrogen and energy are normal in animals bearing Ehrlich ascites tumors whether or not they are treated with AGA. Furthermore, AGA had the same antitumor activity toward the Ehrlich ascites tumor whether the mice had normal or zero protein intake. Thus, decreased nitrogen utilization in the tumor-bearing animal is not necessary for the antitumor

**Table 4**

<table>
<thead>
<tr>
<th>Treatment effect</th>
<th>Normal protein + 0.9% NaCl solution (g)</th>
<th>Normal protein + AGA (g)</th>
<th>Zero protein + 0.9% NaCl solution (g)</th>
<th>Zero protein + AGA (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt change (g/8 days)*</td>
<td>+5.4 ± 0.9</td>
<td>+2.6 ± 2.2</td>
<td>−5.4 ± 0.8</td>
<td>−7.5 ± 0.7</td>
</tr>
<tr>
<td>Ascites on Day 8**</td>
<td>+2.1 ± 0.4</td>
<td>+0.8 ± 0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Survival (days)**</td>
<td>20.5 ± 2.2 (20)*</td>
<td>26.3 ± 1.7 (24)</td>
<td>21.1 ± 2.2 (21)</td>
<td>24 ± 1.9 (24)</td>
</tr>
</tbody>
</table>

*Initial weights were 19 to 22 g; all groups were significantly different from each other: I versus II (p < 0.005); all others (p < 0.001).
**Mean ± S.D.; 8 mice/group.
***Ascites judged by a 0 to 4+ scale. Groups I and II were significantly different from each other (p < 0.0005) and from Groups III and IV (p < 0.001). There was a significant (p < 0.001) effect of AGA treatment on survival. The following pairs of means are significantly different from each other: I versus II (p < 0.025); I versus IV (p < 0.005); III versus IV (p < 0.02).
**Numbers in parentheses, median survival time.
action of AGA. The same conclusion can be made from our clinical observations in children with acute lymphocytic leukemia. In these patients, nitrogen balance could be improved by amino acid supplements (7). In the preclinical and clinical studies of this enzyme, toxicity may have been caused or augmented by protein energy malnutrition. These studies have shown that the nutritional state can be modified without altering the action of AGA. Future studies should reassess the toxicity of this enzyme in animals and patients maintained in a normal nutritional state.

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