Combination Therapy of Animal Tumors with L-Asparaginase and Antagonists of Glutamine or Glutamic Acid

George S. Tarnowski, Isabel M. Mountain, and C. Chester Stock

Divisions of Experimental Chemotherapy and Special Studies, Sloan-Kettering Institute for Cancer Research, New York, New York 10021

SUMMARY

Treatment of the 1-day-old Ehrlich ascites tumor with combinations of selected doses of L-asparaginase and the L-glutamine antagonist, 6-diazo-5-oxo-L-norleucine, or of the L-glutamic acid antagonist, DL-methionine-DL-sulfoximine, produced significantly more growth inhibition without a marked increase in host toxicity than did treatment with L-asparaginase administered alone. In the 3-day-old tumor treated with the combination of L-asparaginase and 6-diazo-5-oxo-L-norleucine, there was a significant enhancement of growth inhibition as compared with the effect of either drug used alone. A less significant increase of growth inhibition was obtained with combination of L-asparaginase and L-glutamyl-γ-hydrazide as compared with the effect of this chemical administered alone.

Combinations of L-asparaginase and 6-diazo-5-oxo-L-norleucine produced increased antitumor effects in mouse melanoma B16 and to a lesser degree in the solid form of Ehrlich tumor and Walker carcinosarcoma 256 of the rat.

Enhanced antitumor activity was seen when 2-deoxy-D-glucose was included with L-asparaginase, azaserine, DL-methionine-DL-sulfoximine, and their combinations in treatment of the Ehrlich ascites tumor.

INTRODUCTION

Growth of certain classes of animal and human tumors can be retarded by treatment with L-asparaginase either from guinea pig serum or from cultures of Escherichia coli (1, 2, 10, 17). Sensitivity of such tumors to this enzyme is caused by the low activity of L-asparagine synthetase which forces them to rely on the external supply of preformed L-asparagine (3, 4, 8, 20). Mammalian L-asparaginase synthetase transfers ammonia or the amide group of L-glutamine to the β-carboxyl group of L-aspartic acid to produce L-asparagine (Chart 1); the enzyme requires ATP and Mg²⁺ ions for full activity (12, 18, 19). If the L-asparaginase synthetase in tumors displaying a higher degree of activity of this enzyme could be suppressed in combination with treatment with L-asparaginase, a greater retardation of the growth of such tumors might be expected than would occur by their treatment with L-asparaginase alone. This would be of therapeutic interest only if the combination result was significantly greater than that due to the agent used in addition to L-asparaginase.

In the present study, Ehrlich ascites tumor was selected from those tumors less sensitive to L-asparaginase in order to facilitate demonstration of effect of combination therapy. Combination experiments were conducted with L-asparaginase and the antagonists, azaserine, DON, 2 L-glutamyl-γ-hydrazide, and MSO. The solid forms of the Ehrlich carcinoma, mouse melanoma B16, and Walker carcinosarcoma 256 in the rat were tested in combination experiments with L-asparaginase and DON.

MATERIALS AND METHODS

Effects of chemicals and their combinations were studied in Ehrlich mouse tumor in its ascites³ and solid forms grown in ICR/HA female mice, melanoma B16 grown in C57BL/6 male mice, and Walker carcinosarcoma 256 grown in Wistar male rats. At the time of tumor implantation, the mice weighed 19 to 22 g and rats weighed 100 to 120 g. The standard therapy

---

1 Supported in part by Grant CA-08748 from the National Cancer Institute, USPHS, and the Elsa U. Pardee Foundation. A portion of these data has been presented at the 60th Annual Meeting of the American Association for Cancer Research, March 23 to 25, 1969 (26). Received August 14, 1969; accepted October 23, 1969.

2 The abbreviations used are: DON, 6-diazo-5-oxo-L-norleucine; MSO, DL-methionine-DL-sulfoximine.

3 The strain of the Ehrlich ascites tumor used in the present study carried viable lactate dehydrogenase-elevating virus as determined through the kindness of Dr. V. T. Riley.
schedule consisted of 6 i.p. injections of chemicals or their combinations, administered to each of a group of 5 tumor-bearing animals once a day beginning with the day following inoculation (the 1st day of tumor growth). In groups receiving combined therapy, the L-asparaginase was injected 0.5 hr after other chemicals. Results were evaluated at the end of therapy by the previously described techniques (22, 27). Animals were given food and water ad libitum.

Chemicals tested were: L-asparaginase from E. coli obtained from the Farbenfabriken Bayer AG (Wuppertal-Elberfeld, Germany), azaserine and DON from the Cancer Chemotherapy National Service Center (Bethesda, Md.), L-glutamyl-γ-hydrazide from Parke, Davis, and Company (Detroit, Mich.), and MSO (Calbiochem, Los Angeles, Calif.) and 2-deoxy-D-glucose (Aldrich Chemical Co., Inc., Milwaukee, Wis.) which were purchased. Chemicals were dissolved in 0.9% NaCl solution; solutions of azaserine, DON, and L-asparaginase were prepared fresh daily.

RESULTS

Experimental designs used in combination therapy of cancer and the methods of evaluation of results have been the subject of a considerable discussion and have been repeatedly reviewed (5, 6, 14, 28). It has been pointed out that the effects of chemicals on both the tumor and its host produced by several doses of chemicals should form the basis of useful experimental protocols for combination therapy. In the present study, combinations included 2 chemicals, one of which was L-asparaginase (X) and the other a glutamine or glutamic acid antagonist (Y); the protocol included the full and quarter-doses of either chemical, their combinations, and the NaCl solution control. Appropriate doses of comparably low levels of toxicity were selected. The full dose was 450 i.u./kg/day for L-asparaginase, 0.1 mg/kg/day for DON (0.05 mg/kg/day in the case of Walker carcinosarcoma 256), 1.25 mg/kg/day for azaserine, 20 mg/kg/day for MSO, and 50 mg/kg/day for L-glutamyl-γ-hydrazide.

Average values of tumor size and host weight for the 3 experiments performed with L-asparaginase and DON in Ehrlich ascites tumor with therapy beginning on the day following inoculation are shown in Chart 2. The data for Chart 2 are also presented in Table 1 as an aid in interpreting the other charts. Effects on Ehrlich ascites tumor of combined therapy starting on the 1st day of tumor growth are graphically demonstrated by plotting the ratios of the total packed ascites cell volumes of treated to diluent control tumors (TIC values) against the relative weight of tumor hosts, i.e., the ratio of host weights at the end to that at the beginning of therapy (Chart 2). In ascites tumors, the host weight at the end of therapy was determined as the weight of mice drained of their ascites. The decrease of average total packed cell volume following therapy with chemicals or their combinations was not attributable to a decrease in size of treated tumor cells since the distribution of cell sizes determined with a Coulter counter did not reveal any shift toward smaller size classes. Small volume or absence of ascites in the peritoneal cavity of treated mice was the main factor contributing to the decrease of the values of average total packed cell volume.

Chart 2. Combined therapy of Ehrlich ascites tumor with L-asparaginase and DON. Results at the end of therapy (6 daily i.p. injections starting on the day after tumor implantation). Doses for L-asparaginase are in i.u./kg/day and for DON in mg/kg/day. - - - - , the lower confidence limit (99.5%) of the total packed cell volume of NaCl solution-treated Ehrlich ascites-bearing control mice, based on the data accumulated during the period of experiment. TPCV, total packed cell volume expressed as the ratio of the average total packed cell volume of the treated group to that of the NaCl solution-treated control groups (T/C). Relative weight = (sum of the average total packed cell volume of the treated group) / (sum of the initial host weights).

Table 1

Combined therapy of Ehrlich ascites tumor with L-asparaginase and DON

Therapy was started on the 1st day after tumor inoculation; 6 i.p. doses, 1/day. Average values for 3 tests; results at the end of therapy.

<table>
<thead>
<tr>
<th>L-Asparaginase (i.u./kg/day)</th>
<th>DON (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>112.5</td>
<td>0.73</td>
</tr>
<tr>
<td>450</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Carass weight change (g)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>112.5</th>
<th>450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2.0</td>
<td>-2.4</td>
<td>-4.5</td>
</tr>
<tr>
<td></td>
<td>+1.4</td>
<td>-1.6</td>
<td>-3.4</td>
</tr>
<tr>
<td></td>
<td>-1.3</td>
<td>-4.6</td>
<td>-4.6</td>
</tr>
</tbody>
</table>

Relative weight

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>112.5</th>
<th>450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.91</td>
<td>0.89</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>1.08</td>
<td>0.91</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>0.94</td>
<td>0.79</td>
<td>0.79</td>
</tr>
</tbody>
</table>

*aSee legend to Chart 2.*
G. S. Tarnowski, I. M. Mountain, and C. C. Stock

It can be seen that all 4 combinations of 2 chemicals produced a very high degree of tumor suppression beyond the effects of L-asparaginase at the cost of a relatively moderate increase of toxicity to the host. Mortality never exceeded 1 out of a group of 5 animals at the end of therapy and occurred only in the control and X/4 groups. Effects of the full dose of DON in tumors treated from the 1st day of growth was so marked that the greater effectiveness of combinations of DON with L-asparaginase in comparison with the effects of single chemicals was evident only in combinations including the quarter-dose (0.025 mg/kg/day) of DON.

On the other hand, in tumors the therapy of which started on the 3rd day of tumor growth and consisted of only 4 instead of 6 injections, all 4 combinations of the 2 chemicals produced a significantly greater degree of tumor growth inhibition than did the high dose of either chemical administered alone (Table 2 and Chart 3). Moreover, in this case, there was no additional toxicity in the hosts treated with combinations of drugs.

Combined therapy with L-asparaginase and DON of the Walker carcinosarcoma 256 of the rat produced a significant degree of tumor growth inhibition (p < 0.05) when therapy started on the 1st day of tumor growth (Table 2). Combined therapy did not cause an additional decrease of relative host weights. Neither chemical administered alone in the doses used for combined therapy has produced a significant tumor inhibition.

Treatment with combinations of L-asparaginase and DON of mouse melanoma B16 produced a significant inhibition of tumor growth (Table 2). Neither chemical administered alone produced an antitumor effect. In the solid form of Ehrlich tumor some additional inhibition was induced by the combination as compared to either chemical alone (Table 2).

In Ehrlich ascites tumor, the effect of combined therapy with L-asparaginase and azaserine was not greater than the effect of the full dose of either chemical alone when therapy started on the 1st day of tumor growth (Chart 4, Table 2). On the right side of Chart 4, the effect is shown of a triple combination of L-asparaginase, azaserine, and 2-deoxy-D-glucose on the growth of Ehrlich ascites tumor treated from the 1st day of growth. 2-Deoxy-D-glucose is an inhibitor of the utilization of glucose by tumor cells (7); it diminishes the intracellular level of ATP, 1 of the participants in the biosynthesis of L-asparagine (13, 16). Administered alone in a dose of 200 mg/kg/day, 2-deoxy-D-glucose decreased the

Table 2

<table>
<thead>
<tr>
<th>Tumor</th>
<th>DON</th>
<th>Azaserine</th>
<th>MSO</th>
<th>Glutamyl-γ-hydrazide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>EA, 1 day</td>
<td>p &lt; 0.001</td>
<td>N.S.</td>
<td>N.S.</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>EA, 3 day</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>N.S.</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>EC</td>
<td>p &lt; 0.05</td>
<td>N.S.</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>B16</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>W256</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

aEA, 1 day: Ehrlich ascites tumor; treatment began on the 1st day of tumor growth (the day after implantation). EA, 3 day: Ehrlich ascites tumor; treatment began on the 3rd day of tumor growth. EC, Ehrlich tumor in solid form. B16, Melanoma B16. W256, Walker carcinosarcoma 256.

bA, both versus L-asparaginase.

cB, both versus other chemical: DON, azaserine, MSO, or glutamyl-γ-hydrazide.

dN.S., not significant, p > 0.05.
average total packed cell volume of Ehrlich ascites tumor cells. Combinations of 2-deoxy-D-glucose with L-asparaginase, azaserine, and their combinations produced even greater antitumor effects. Increase of the dose of 2-deoxy-D-glucose to 400 mg/kg/day did not cause an additional augmentation of either antitumor or toxic effects of the 2 other chemicals.

In combination with MSO, L-asparaginase produced a markedly increased inhibition of growth of Ehrlich ascites tumor but also caused greater toxicity as evidenced by decreased relative weights of treated hosts (Chart 5). 2-Deoxy-D-glucose augmented antitumor effect of L-asparaginase administered alone but did not increase the antitumor effects of MSO or combinations.

Combined therapy of Ehrlich ascites tumor starting the day after inoculation and using L-asparaginase and L-glutamyl-γ-hydrazide did not produce a significant increase of antitumor effect in comparison with the effect of L-asparaginase alone; however, the effect of combinations was significantly greater than that of L-glutamyl-γ-hydrazide administered alone (Table 2).

**DISCUSSION**

Results of the present study with several animal tumors have demonstrated the greater effectiveness in certain combinations of L-asparaginase and 4 different antagonists of L-glutamine or L-glutamic acid compared with the antitumor effects produced by each chemical administered alone. Other investigators (9, 15) have reported prolongation of the survival time of mice bearing a lymphosarcoma or lymphatic leukemias when such animals were treated with combinations of L-asparaginase (E. coli 2) with azaserine or azotomycin. All these data seem to be in accord with the assumption that L-glutamine antagonists augment the therapeutic effects of L-asparaginase by competing with L-glutamine for the L-asparagine synthetase in tumor cells which are relatively resistant to L-asparaginase. Several observations indicate, however, that the underlying mechanisms of action are more complex. In selected doses, DON in combination with L-asparaginase has produced a significant enhancement of the inhibition of Ehrlich ascites tumor whereas azaserine did not. Differences in pathological changes produced in rats and mice by DON and azaserine, respectively, have been described (24, 25). Both chemicals damage bone marrow and intestinal mucosa but only azaserine produces necrosis in the pancreas, liver, and kidney. DON, but not azaserine, produced in mice pronounced cumulative effects. The cumulative LD50 dosage for 5 daily i.p. injections of DON is 1% of the LD50 for a single i.p. injection. Acute toxicity of DON is 30 to 40 times greater than that of azaserine. This difference may to some extent be attributable to the presence in normal and tumor tissues of rats and mice of an enzyme deaminating azaserine but not DON (21); however, it has been shown that, in the *in vitro* conversion of formylglycinamide ribotide to formylglycinamidine ribotide, DON is required in only one-fortieth the concentration of azaserine to give an equivalent inhibition (11).

The differences in biological properties of the 2 glutamine antagonists should not be surprising. The amide group of glutamine is used as the source of nitrogen in the synthesis not only of L-asparagine but also of purines, cytosine, glucosamine, tryptophan, and nicotinamide (23). Different glutamine antagonists presumably interfere to a different extent with these biosynthetic reactions and the biological consequences of such an interference might differ from tissue to tissue. These biosynthetic reactions require ATP as one of the participants, and it was hoped that 2-deoxy-D-glucose, which can diminish the level of ATP in cells, would augment the antitumor effects of L-asparaginase and azaserine.

Studies of the effects of glutamine antagonists on the L-asparaginase synthetase activity of the supernatant fraction of sonically disrupted Ehrlich ascites tumor cells are being conducted in this laboratory. Preliminary results have shown that DON and azaserine, but not MSO, inhibit the activity attributable to the utilization of added L-glutamine as the source of amide nitrogen (unpublished data). Patterson and Orr (19) reported that azaserine moderately inhibits the activity of purified L-asparagine synthetase isolated from the
Novikoff hepatoma, but they did not examine the effects of DON, MSO, and L-glutamyl-γ-hydrazide.

Another complication in the utilization of glutamine antagonists for treatment of tumors in combination with L-asparaginase is introduced by the fact that, in addition to glutamine, L-asparagine synthetase can utilize un-ionized ammonia as the source of amide nitrogen (19). Further studies observed differences in antitumor and other biological activities of L-glutamine and L-glutamic acid antagonists administered alone and in combination with L-asparaginase.

ACKNOWLEDGMENTS

We thank Mrs. Hannah E. Perutz and Messrs. Stephen E. Banks, Michael H. Perlman, and Edward M. Solney for assistance in the execution of chemotherapeutic tests.

REFERENCES


Combination Therapy of Animal Tumors with l-Asparaginase and Antagonists of Glutamine or Glutamic Acid

George S. Tarnowski, Isabel M. Mountain and C. Chester Stock


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/30/4/1118

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.