Enzymatic Deamination of 8-Azaguanine in Normal and Neoplastic Tissues*†

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In 1949, Kidder and collaborators (9) demonstrated that 8-azaguanine strongly inhibited the growth of several transplanted mouse tumors. Following this report, the effect of 8-azaguanine on the growth of many experimental neoplasms has been studied in this and other laboratories (1, 4, 6, 10, 12, 17–19). With one exception, the August carcinoma, all rat tumors were resistant to the carcinostatic action of this compound. On the other hand, the only rabbit tumor which has been studied, the Brown-Pearce carcinoma, was significantly inhibited. In the mouse, the species of choice for most of the investigations, this chemo- therapeutic agent was effective against a variety of carcinomas and certain leukemias but not against other leukemias, melanomas, and sarcomas.1

The present investigation was undertaken in the hope of finding a reason for this striking dichotomy between azaguanine-susceptible and azaguanine-resistant tumors. It had been established in preliminary experiments that an enzyme system present in mouse and rabbit tissues catalyzed the deamination of 8-azaguanine to 8-azaxanthine (5,7-dihydroxy-1H-v-triazolo [dl pyrimidine] (5); proof of the identity of the end-product of this deamination as 8-azaxanthine, by filter paper chromatography and comparative ultraviolet spectroscopy, has been recently described (11).

More extensive data on the deamination of 8-azaguanine by a guanase preparation were reported by Roush and Norris (15). The data to be presented suggest that the activity of this enzyme is an important factor in determining the response of various tumors to 8-azaguanine.

MATERIALS AND METHODS

Experimental material.—The following transplantable tumors were employed: Sarcoma 180 in Paris RIII or C57 mice; mammary adenocarcinomas 755 and E 0771 in C37 mice; mammary adenocarcinoma RC in DBA mice; lymphosarcoma 6CSHED in CSH mice; myelogenous leukemia C1498 in C37 mice; Brown-Pearce carcinoma in rabbits. The details of tumor transplantation, response to 8-azaguanine, and tumor growth measurements have been reported previously (6). For the present experiments, tumors were allowed to grow without treatment until they reached a suitable weight without evidence of necrosis; only healthy, non-necrotic tissue was used. Each sample represented a pool of tissue from two to five rabbits or ten to twenty mice.

Normal tissues were obtained from both normal and tumor-bearing mice and rabbits. The livers were routinely pooled after removal of the gallbladder and perfusion with or washing in cold saline. Animals were killed with chloroform or nembutal; it was ascertained in preliminary experiments with other methods of killing, i.e., air injection or decapitation, that anesthetic agents had no effect on the azaguanine deaminase activity of normal or tumor tissue.

Almost without exception, analyses were carried out immediately after removal of the tissue from the animals; however, rapid freezing of the tissues in a deep-freeze, followed by slow thawing at room temperature at a later time, did not appear to cause any decrease in enzyme activity.

Preparation of homogenates.—The pooled tissue was cut into small pieces, and a weighed sample was homogenized for 15 minutes in a Potter all-glass homogenizer with hypertonic (0.77 M) sucrose in 0.01 M phosphate buffer at pH 7.2. The final concentration of tissue in the homogenate was routinely 15 per cent on a wet weight basis. All operations were carried out at a temperature ranging from 0 to 4°C.

The homogenate was subdivided into aliquots for nuclear count, determination of azaguanine deaminase activity, and other analyses of interest.

Determination of azaguanine deaminase activity.—Enzyme activity was assessed by determination of initial linear reaction rates on the basis of a microdetermination of the ammonia evolved (16) when the homogenate was incubated with substrate for varying periods of time. The procedure is, in brief, as follows: 1 ml of homogenate (= 150 mg fresh tissue) was incubated with 1 ml of 8-azaguanine solution (= 5 mg 8-
azaguanine) in a small side-arm test tube maintained at 37°C in a water bath. A fresh substrate solution was prepared each time by suspending the required amount of 8-azaguanine in half the required volume of distilled water and dissolving it by the addition of one or two drops of 80 per cent NaOH; the resulting solution was adjusted to pH 8.0 with 2 N HCl and diluted to mark with water.

The enzyme reaction was stopped by the addition of 3 drops of 8 N H2SO4. Each incubation tube was then connected by means of rubber tubing to another side-arm test tube containing 2 ml. of 2 per cent H2BO3 + 0.02 ml. of indicator (8 parts per cent brom cresol green and 1 part 0.1 per cent methyl red in 95 per cent ethanol). The incubation mixture was made alkaline with 1 ml. of half-saturated K2CO3, and the ammonia liberated was aspirated for 20 minutes into the boric acid solution. The latter was back-titrated to its original pink color with standard dilute H2SO4 in a 1-nil. total capacity ultramicroburette (E. Greiner & Co.) of modified design.

Control tubes in which 1 ml. H2O was substituted for the substrate solution were incubated for the same length of time and provided a measure of the blank caused by non-specific ammonia production in the homogenates. All values were corrected for the corresponding blanks and expressed as μg ammonia nitrogen/hour/ml homogenate. From these values, deaminase rates were calculated on a cell basis (μg ammonia nitrogen/hour/106 cells).

Calculation of results on a cell basis.—Almost all the results of studies on the biochemical composition of various tissues in the past have been expressed in the basis of nitrogen or units of fresh or dry weight of tissue. Recently, Davidson and Leslie (9, 13) and Price and Laird (18) have demonstrated the advantages of using the number of cells in a sample as the basic unit of calculation in analyses of intracellular components. This concept has been applied here; an evaluation of this approach and a report on the requisite methods and on results obtained on a variety of normal and neoplastic tissues will be presented elsewhere.

The number of cells in a sample of tissue prepared for determination of azaguanine deaminase activity was ascertained by direct enumeration of nuclei in an aliquot of the same homogenate. For this purpose, duplicate 1-ml. aliquots were diluted (1:60) with 8 per cent acetic acid containing 0.02 per cent methyl green, and the nuclei in each final suspension were counted in duplicate in a hemacytometer (14). It was assumed that each nucleus represented one cell. It is, of course, recognized that the results obtained by this general procedure represent average values for the various kinds of cells which are present in each tissue.

RESULTS

Properties of the enzyme preparations.—It has been pointed out that the determination of azaguanine deaminase in tissue homogenates is based on the initial linear reaction rate. Chart 1 demonstrates that the rate of evolution of ammonia nitrogen in homogenates of different activity is a linear function of time and illustrates the type of experimental data upon which the present report is based. Homogenates of tissue with a high deaminase activity were routinely incubated for 30, 60, and 90 minutes, whereas the incubation period was prolonged to 180 minutes for homogenates of low activity.

Experiments on the effect of dilution of homogenate on the rate of enzymatic deamination of 8-azaguanine demonstrated that this rate was proportional to the amount of tissue present in the homogenate.

Preliminary experiments on the localization of azaguanine deaminase within the cell indicate that the enzyme is not associated with the nuclear fraction.

Lack of carcinostatic activity of 8-azaanthraine.—The effect of 8-azaanthraine on a variety of transplanted tumors was investigated with the same methods described in the experiments with 8-azaguanine (6). The appreciably greater solubility of the dihydroxy derivative and its lack of toxicity to mice at daily doses as high as 500 mg/kg permitted the administration of 4—6 times as great an amount of 8-azaanthraine as of 8-azaguanine. Nevertheless, no inhibition of growth of any of the tumors under investigation was observed (Table 1).

In the enzymatic deamination of 8-azaguanine, therefore, a compound which was strikingly carcinostatic for four of the seven tumors under investigation was converted to a derivative which failed to exhibit any inhibitory activity against any of the seven tumors.

Azaguanine deaminase activity of normal tissues.—Table 2 summarizes the data obtained on various tissues of normal and tumor-bearing mice and rabbits. In mice, the values ranged from the low deaminase activity of kidney to the extremely high activity in intestine;4 among the rabbit tissues...

1 Lederle 8-azaguanine, batch no. 7-7242, was used in these experiments; ultraviolet spectroscopic analysis indicated a purity of 85 per cent. In preliminary experiments, a pure sample of 8-azaguanine obtained through the courtesy of Dr. Morris Engelman was used for comparative purposes.

2 Manuscript in preparation.

3 For these determinations, the small intestine was used after removal of the intestinal contents. A separate experiment on the latter indicated that the small amounts of intestinal contents which might still have been present in the homogenate might have been responsible for less than 5 per cent of the total deaminase activity of this tissue.

4 Manuscript in preparation.
investigated, only liver and intestinal mucosa exhibited measurable activity.

Experiments with blood were carried out in both species. The blood was allowed to clot; the clot was briefly homogenized and subjected to freezing and thawing. In a typical experiment, 1 ml. of mouse blood caused the production of more than 400 μg. ammonia nitrogen in the first hour (100 per cent deamination = 620 μg. ammonia nitrogen). The enzymatic activity of the corresponding serum was very low; this finding indicated that azaguanine deaminase was associated with the erythrocytes. There was no measurable activity in rabbit blood or serum.

In view of the significant deaminase activity of mouse blood, it was of interest to determine whether the amount of blood remaining in the tumors upon their removal from the animals represented an appreciable source of in vitro deaminase in these homogenate experiments. A pooled sample of 755 tumor, the mouse neoplasm with the lowest deaminase activity, was homogenized in distilled water, and the hemoglobin content of the supernatant fluid after prolonged high-speed centrifugation (32,000 × g) was determined. Suitable calculations from the hemoglobin content of this homogenate led to the conclusion that the amount of blood remaining in the tumor after the death of the host could not have been responsible for more than 10 per cent of the deaminase activity of this tumor. The hemoglobin content of a similar homogenate of Sarcoma 180 was negligible.

AZAGUANINE DEAMINASE ACTIVITY OF NORMAL TISSUES

Table 1 presents the data obtained with azaguanine-susceptible and azaguanine-resistant tumors. The following observations may be made: (a) the Brown-Pearce tumor appears to be qualitatively different from the other tumors, since it does not have measurable azaguanine deaminase activity, even when the time of incubation was extended to 5 hours; (b) in homogenates of the three tumors resistant to 8-azaguanine the in vitro deamination of the carcinostatic agent to an inactive derivative proceeds at a rapid rate; (c) homogenates of three of four tumors susceptible to the drug exhibit a low or negligible deaminase activity. It may, then, be postulated that azaguanine deaminase activity and a pronounced susceptibility to the drug play a part in determining the response of a particular tumor to the drug.

This hypothesis does not seem to hold for the E 0771 tumor, which exhibits both a high azaguanine deaminase activity and a pronounced susceptibility to the drug. At the present time we have no evidence to explain this discrepancy.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>No. of Experiments</th>
<th>Av. Deaminase Rate (μg NH₃-N/hour/10⁶ cells)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>Liver</td>
<td>4</td>
<td>2.42</td>
<td>1.00–2.92</td>
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<tr>
<td></td>
<td>Kidney</td>
<td>3</td>
<td>0.56</td>
<td>0.35–0.70</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>3</td>
<td>4.18</td>
<td>1.81–5.57</td>
</tr>
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<td>Tumor-bearing mice*</td>
<td>Liver</td>
<td>17</td>
<td>2.48</td>
<td>1.55–4.88</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>2</td>
<td>&gt;16</td>
<td></td>
</tr>
<tr>
<td>Normal rabbits</td>
<td>Liver</td>
<td>4</td>
<td>5.06</td>
<td>2.20–4.34</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>2</td>
<td>0†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intestinal</td>
<td>1</td>
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<td></td>
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<tr>
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<td>Mucosa</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Testis</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tumor-bearing rabbits</td>
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<td>1.84</td>
<td>0.61–3.88</td>
</tr>
<tr>
<td></td>
<td>Testis</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* All groups of mice, regardless of the identity of the tumor present, are included.
† No measurable deamination in 180 minutes.
In the experiments summarized in this report, each tumor was obtained from a different group of mice. It was of interest to ascertain whether the presence of an azaguanine-resistant tumor in the same hosts with an azaguanine-susceptible tumor exerted any effect on the deaminase activity of the latter and vice versa. In one experiment, Sarcoma 180 and the 755 tumor were implanted into the same mice, the resistant tumor into one axilla and the susceptible tumor into the other. The azaguanine deaminase activities of Sarcoma 180 and 755 tumor in this experiment (cf. Chart 1) were 0.94 and 0.30 μg ammonia nitrogen/hour/10⁸ cells, respectively (average of all samples of Sarcoma 180 and 755 tumor: 0.98 and 0.27 μg ammonia nitrogen/hour/10⁸ cells, respectively). The presence of a second tumor in the same hosts, then, had no effect on the deaminase activity of either tumor.

DISCUSSION

The results of this investigation, which was based in its entirety on the measurement of in vitro deamination of 8-azaguanine by tissue homogenates, lead to certain inferences concerning the in vivo action and metabolism of this chemotherapeutic agent. The data which have been reported are consistent with the following conclusions: (a) a major portion of any therapeutic dose of azaguanine following injection into the tumor-bearing animals is rapidly deaminated by liver, brain, and other organs and thus rendered inactive as a carcinostatic agent; (b) an increasingly minute portion of the drug reaches the cells of the tumor as this “detoxification” in the normal tissues continues; (c) the cells of some of the tumors have a sufficiently high azaguanine deaminase activity so that the intracellular level of the drug is kept below the threshold concentration needed for inhibition of tumor growth, whereas the deaminase activity of the cells of other tumors is too low to afford them this protection. The clear-cut dichotomy in the susceptibility of various tumors to 8-azaguanine could be explained on this basis.

In a concomitant study in this laboratory (7), it has been demonstrated by means of filter paper chromatography and quantitative ultraviolet spectroscopy that 8-azaguanine is very rapidly deaminated in vivo to 8-azaxanthine and that both compounds are rapidly excreted. The metabolic significance of the high rates of in vitro deamination of 8-azaguanine by homogenates of normal tissues has thus been confirmed. It cannot yet be decided whether the observed difference in azaguanine deaminase activities between azaguanine-susceptible and azaguanine-resistant tumors constitutes the only factor which determines the response of a tumor to the drug or whether it provides only a partial answer. In view of the findings with mammary carcinoma E 0771, the second alternative is the more likely one. The available evidence indicates, however, that enzymatic control of cellular levels of 8-azaguanine is an important factor in the success or failure of tumor chemotherapy with this drug.

SUMMARY

1. The enzymatic deamination of 8-azaguanine by homogenates of normal and neoplastic tissues of mice and rabbits has been studied.
2. This reaction transforms 8-azaguanine, which inhibits the growth of four of the seven tumors under investigation, into 8-azaxanthine, which has no carcinostatic activity for any of the seven tumors.
3. The distribution of azaguanine deaminase in normal tissues has been reported; the activity of liver and other tissues is sufficiently high to cause the rapid inactivation of major portions of injected 8-azaguanine.
4. The azaguanine deaminase activity of the three azaguanine-resistant tumors is high; that of three out of four azaguanine-susceptible tumors is low or negligible.
5. The significance of these findings for an assessment of the different response of different tumors to 8-azaguanine has been discussed.

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REFERENCES


* Full manuscript in preparation.
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