Enzymatic Deamination of 8-Azaguanine in Normal Human Brain and in Glioblastoma Multiforme*

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In experimental cancer chemotherapy, attention has been focused for some time on the guanine analog, 8-azaguanine. Extensive tests in various laboratories have shown that this agent inhibits the growth of certain experimental neoplasms but fails to affect the growth of others. In a recent report from this laboratory (3), it was demonstrated that an enzyme, azaguanine deaminase, is present in normal and neoplastic tissues of mice and rabbits which catalyzes the deamination of the tumor inhibitor, 8-azaguanine, to a completely inactive derivative, 8-azaxanthine. The rate of deamination is significantly higher in homogenates of normal tissues and of three azaguanine-resistant tumors than in homogenates of three of the four azaguanine-susceptible tumors which were studied. It was postulated that enzymatic control of cellular levels of 8-azaguanine is an important factor in the success or failure of tumor chemotherapy with this drug.

Naturally, it was of interest to extend this investigation to a suitable human tumor. In the course of a study of certain metabolic aspects of human brain tumors of the general type glioblastoma multiforme, it was observed that 8-azaguanine has an inhibitory effect on these tumor cells cultured in vitro, while 8-azaxanthine does not affect their growth and development in any way. The first section of the present report briefly summarizes the evidence upon which this conclusion was based; a fuller description of this and related findings is in preparation.† In the second section of this report, comparative analyses for azaguanine deaminase in homogenates of normal brain and glioblastoma are given, together with chromatographic evidence which highlights the relevance of these in vitro findings for the behavior of glioblastoma cells in tissue culture in the presence of this carcinostatic agent.

METHODS AND RESULTS

TISSUE CULTURE EXPERIMENTS

Materials and methods.—A 0.1 M stock solution of 8-azaguanine was prepared by heating a suspension of the solid in one-third the final volume of 0.5 N NaOH until a clear solution was obtained. This solution was then partially neutralized with 1 N HCl and made up to volume. A stock solution of 8-azaxanthine was prepared in the same way. The stock solutions were incorporated into the experimental feeding solutions so as to produce final concentrations of 0.002–0.005 M. A control solution containing NaOH alone at the concentration present in the 0.005 M 8-azaguanine solution was included in each experiment.

Glioblastoma specimens from fifteen different patients were tested in a series of tissue culture experiments with 8-azaguanine. For normal tissue controls, it would have been ideal to use normal human adult glial tissue. However, because of its relatively high content of intercellular material and the consequently slow and spotty character of its growth, and in view of the fact that it cannot be transferred from tubes to slides, its use for this purpose was not practical. In this series of experiments, therefore, the non-neoplastic tissue controls consisted of stock cultures of neuroglia from normal fetal brain, obtained at hysterotomy in approximately the third month of gestation, and carried in roller tubes until needed. The data to be presented indicate that this tissue does not constitute a true negative control, as far as the action of 8-azaguanine is concerned.

General summary of results.—The detailed observations on the two types of tissue will be presented elsewhere.‡ The following conclusions were drawn from the entire series of experiments: (a) Concentrations of 8-azaguanine from 0.02 to 0.002 M produced varying degrees of damage to tumor tissues and to fetal brain. (b) Only in the highest

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concentration were these effects exerted immediately, and then only on the tumor tissues. The fetal glia usually exhibited a response to the drug at least 24 hours later than the tumors, were somewhat less damaged at the same concentration of the agent, and made a better recovery after withdrawal when the exposure had not been sufficient to kill the cells. Concentrations of 0.01 M, 0.005 M, and 0.002 M came to be used routinely, with a 5–10-day period of exposure. 8-Azaguanine at 0.01 M was lethal in this period of time to most tumor tissues but was usually not completely lethal to the fetal glia. (c) The growth rate of the exposed tissues seemed to influence their susceptibility to the inhibitor. The tumors used in these experiments included glioblastomas, astrocytomas, and spongioblastomas. Of these, the most rapidly growing tumors, whether of the glioblastoma or the astrocytoma type, were the most severely affected. Of the normal fetal brain tissues, oligodendroglia were the most prolific type, and these were also the most severely damaged. It appears, therefore, that the inhibitory effects of 8-azaguanine are exerted to the greatest extent on rapidly growing tissues, whether neoplastic or normal, and that the extent of the damage is roughly parallel to the growth rate.

Detailed findings.—These observations may be illustrated by a description of the results of one experiment in which the action of 8-azaguanine and 8-azaxanthine on a glioblastoma and a sample of fetal brain was investigated. The tumor used in this experiment was a glioblastoma of type multiforme (J. McG. 44, Unit No. 03-027-79, Feb. 28, 1951), which produced in vitro a variety of cell types, including spindle-shaped spongioblasts, astroblasts, and some oligodendroblasts. Its growth rate, in the setting of all the glioblastomas with which we have had experience, would be classed as median. This tumor was carried as stock for 14 days in roller tubes handled according to the method of Gey and Gey (2). At the end of this time, it was explanted to slides, by an adaptation of the Maximow double coverslip method (5), with a basic medium composed of human placental serum, beef serum ultrafiltrate, chicken embryo extract, and chicken plasma, in which the drugs were incorporated at the time of explantation so as to be present in final concentrations of 0.005 M or 0.002 M.

The solutions remained in contact with the tissues for 7 days; during this time the cultures were washed once in isotonic saline (balanced and buffered) and exposed again to the same medium, from which only the plasma was omitted. The slides were observed daily at magnification × 800. At the end of this time, one-half the cultures were fixed and stained with Harris’s hematoxylin or with Mallory’s phosphotungstic acid hematoxylin. The other half were washed as before and returned to normal feeding solution, from which the drug was now omitted. A total of 35 tumor slides, each carrying two explants, were employed.

The sample of fetal brain was explanted to 30 slides with two explants each, which were handled at the same time and in the same manner as the tumor slides. Table 1 summarizes the arrangement of this typical experiment.

The following findings were made: No damage was observed in any cultures except those exposed to 8-azaguanine. The series treated with 8-azaxanthine exhibited growth which was at least as good as that of the controls, if not better, and showed no intracellular signs of damage. No adverse effect upon the tissues attributable to pH could be detected when these alkaline solutions were introduced into the culture medium, buffered as it was with serum; the agent did not precipitate out at these concentrations in this buffered medium.

At the end of a 7-day exposure to 0.005 M 8-azaguanine, cultures of this glioblastoma had responded with (a) marked inhibition of migration from the explant (as compared to the extent of cellular migration in the untreated controls), (b) development of basophil cytoplasmic granules, (c) some nuclear damage (fragmentation, pyknosis, etc.), (d) inhibition of mitosis, and (e) rounding up of many cells which normally would be extended, and disintegration of some cells. Tumor cultures exposed to 0.002 M concentrations of the agent showed clear evidence of damage on all these five counts, but to a lesser degree than those exposed to 0.005 M 8-azaguanine.

Cultures of fetal brain exposed to the higher level of the drug were damaged in a similar manner
as the tumor cultures but in varying degrees, with explants from the parietal and temporal areas most severely affected and those from the medul- lary region responding only slightly to the drug. At the lower level, the same general changes took place, but with a lower peak of damage.

After withdrawal of the experimental agents at the end of 7 days and the return of the cultures to normal feeding solutions, both fetal and neo- plastic tissues recovered completely; the tumor, however, required a distinctly longer time than the embryonic tissue to effect this complete recovery.

Homogenate Experiments

Materials and methods.—Tissues for the in vitro determina- tion of azaguanine deaminase activity were obtained directly from the operating rooms at Neurological or Psychiatric Institute. Uniform precautions were taken to maintain all samples near O° C. until they were transferred to a deep-freeze unit at —20° C. for storage. Preliminary experiments had shown that the activity of this enzyme after storage is the same as in fresh tissues.

The tumors were all classified as glioblastoma multiforme. The non-neoplastic samples of human brain were obtained either from topectomies or from the excision of noncancerous areas of the brain required to reach the site of a glioblastoma.

The procedure employed for the determination of azagua- nine deaminase activity in homogenates of these tissues has been described in detail (3). All activities were calculated on a cell basis, as in the previous investigation. It should be pointed out that, while the glioblastomas yielded homogenates in which the number of nuclei could be determined in a re- producible and unequivocal way, the estimation of the nuclear count in homogenates of non-neoplastic samples of brain was somewhat less reliable, probably because of the high lipid content of these samples.

Results.—The comparative azaguanine deami- nase activity of neoplastic and non-neoplastic samples of human brain is detailed in Table 2. None of the glioblastomas which have been studied exhibited any measurable deaminase activity by this method. The values obtained on non-neo- plastic human brain are higher than those of normal rabbit and mouse tissues (3); it may be re-called that in the mouse the highest activity was cap- able of carrying out the conversion of 8-aza- guanine to the inactive derivative, 8-azaxanthine, at a very appreciable rate, while the glioblastomas yielded homogenates in which the number of nuclei could be determined in a reproducible and unequivocal way, the estimation of the nuclear count in homogenates of non-neoplastic samples of brain was somewhat less reliable, probably because of the high lipid content of these samples.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Azaguanine Deaminase Rate (NEX·N/hour/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. B.</td>
<td>Normal brain*</td>
<td>5.8</td>
</tr>
<tr>
<td>A. T.</td>
<td>Normal brain</td>
<td>12.6</td>
</tr>
<tr>
<td>T. C.</td>
<td>Noncancerous area†</td>
<td>&gt; 4.8</td>
</tr>
<tr>
<td>K. H.</td>
<td>Noncancerous area†</td>
<td>6.0</td>
</tr>
<tr>
<td>L. S.</td>
<td>Glioblastoma</td>
<td>0§</td>
</tr>
<tr>
<td>R. A.</td>
<td>Glioblastoma†</td>
<td>0</td>
</tr>
<tr>
<td>R. A.</td>
<td>Glioblastoma†</td>
<td>0</td>
</tr>
<tr>
<td>F. C.</td>
<td>Glioblastoma</td>
<td>0</td>
</tr>
<tr>
<td>H. K.</td>
<td>Glioblastoma</td>
<td>0</td>
</tr>
<tr>
<td>H. B.</td>
<td>Glioblastoma</td>
<td>0</td>
</tr>
</tbody>
</table>

* Obtained at topectomy.
† Removed to provide access to a glioblastoma.
‡ Two areas of the same tumor.
§ No measurable deamination in 180 minutes.

Experiments with Filter Paper Chromatography

The data demonstrating the inhibitory effect of 8-azaguanine and the lack of activity of 8-azaxan- thine on human glioblastomas were obtained in tissue culture. The information on the compar- rative azaguanine deaminase activity of non-neo- plastic human brain and glioblastoma was gathered by homogenizing tissues made available di- rectly from the operating room. It was of im- portance to correlate these findings and to deter- mine whether the results obtained in homogenates had any relevance to the metabolism of 8-aza- guanine in tissue culture.

For this purpose, feeding fluids which had bathed the explants for several days and which originally contained 8-azaguanine at a concentra-
a less strongly fluorescent background. The $R_f$ values for the two compounds were identical with the values previously published (4, 6). After drying, the separated zones were cut and extracted with 4-ml portions of 0.1 N HCl for 24 hours at 37° C.

Feeding fluids without 8-azaguanine, incubated simultaneously with explants of the same tissue and then chromatographed in an identical manner, served as blank zones. These zones, after extraction with 0.1 N HCl, provided the necessary blank solutions for ultraviolet spectroscopy. The previously published principles for the separation and estimation of micro-amounts of purines and pyrimidines were applied with some modification to the spectroscopic analysis of these extracts in a Beckman quartz spectrophotometer. A solution containing known amounts of 8-azaguanine and 8-azaxanthine was chromatographed as part of each experiment. The ultraviolet absorption spectra of both compounds after isolation from the feeding fluids were routinely checked and found to be identical with those of the standard solutions.

The subsequent computations were based on the differences ($\Delta$) between the extinction ($E$) at a maximum (250 m$\mu$ for 8-azaguanine and 265 m$\mu$ for 8-azaxanthine in 0.1 N HCl) and the extinction at 295 m$\mu$. These $\Delta E$ values were then compared to the $\Delta E$ values obtained for the corresponding standards.

Evaluation of the method.—In eight experiments, the $\Delta E$ values obtained after chromatography and extraction of standard solutions containing 10 $\mu$g/ml of 8-azaguanine and 8-azaxanthine, respectively, were as follows:

- 8-azaguanine $\Delta_{295\text{m}\mu} = 0.380 \pm \text{a.d. } 0.018$
- 8-azaxanthine $\Delta_{295\text{m}\mu} = 0.230 \pm \text{a.d. } 0.025$

In four experiments, known amounts of 8-azaguanine and 8-azaxanthine were added to feeding fluids which had not contained 8-azaguanine throughout the experiment. The recoveries of these substances after paper partition chromatography of this test system were essentially quantitative (100 per cent for 8-azaxanthine and 99 per cent for 8-azaguanine).

Since a known amount of 8-azaguanine was included in the feeding fluids at the beginning of each tissue culture experiment, it was possible to assess the completeness of recovery of this drug either as unchanged 8-azaguanine or 8-azaxanthine by adding the levels of the two compounds estimated at the end of the incubation period. In a total of 22 experiments, the average recovery of the drug in terms of the sum of these two compounds averaged 98 ± a.d. 19 per cent. This finding demonstrated that 8-azaguanine in tissue culture with explants of these tissues was subject essentially to a single metabolic reaction, viz., deamination to 8-azaxanthine.

The expression $\Delta_{295\text{m}\mu}$ indicates the difference in extinction between these two wavelengths. These standard solutions were read against extracts of an absolute paper blank which had been subjected to chromatography.

Results.—Three samples of glioblastoma and two samples of human fetal brain were incubated with feeding fluids in tissue culture for 1–2 weeks; the feeding fluid was renewed every 3–4 days, and all the consecutive feeding fluids were subjected to chromatographic analysis. Table 3 summarizes the results obtained with the tumors, and Table 4 the findings with fetal brain.

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation Period (Hours)</th>
<th>8-Azaguanine Remaining after Incubation (Per cent)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>81</td>
</tr>
<tr>
<td>3†</td>
<td>72</td>
<td>87</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>74</td>
</tr>
<tr>
<td>5</td>
<td>96</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>96</td>
<td>96</td>
</tr>
</tbody>
</table>

* Per cent 8-azaguanine remaining = \[\frac{\mu g \text{ 8-azaguanine}}{(\mu g \text{ 8-azaguanine} + \mu g \text{ 8-azaxanthine})} \] X 100.

† Samples 2 and 3 are portions of tumors also assayed for 8-azaguanine deaminase by the homogenate technic; they correspond to the last two entries in Table 1 (H. K. and H. B.).

### Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation Period (Hours)</th>
<th>8-Azaguanine Remaining after Incubation (Per cent)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>96</td>
<td>38</td>
</tr>
</tbody>
</table>

* Per cent 8-azaguanine remaining = \[\frac{\mu g \text{ 8-azaguanine}}{(\mu g \text{ 8-azaguanine} + \mu g \text{ 8-azaxanthine})} \] X 100.

It may be seen that in the first two samples of glioblastoma, which exhibited the typical severe damage caused by incubation with 8-azaguanine, very small amounts of this agent were converted to the inactive derivative in 48–96 hours. The third glioblastoma, which was not so severely damaged, was able to inactivate a larger proportion of the drug. Fetal brain, which was less affected by 8-azaguanine and which recovered much
more rapidly and completely, brought about the deamination of almost the entire amount of the agent in 72–96 hours; the second experiment in Table 4 demonstrates that this amount of deamination actually required at least a 3-day period.

In view of these lengthy incubation periods, it is evident that the rate of deamination of 8-aza-guanine in tissue culture per hour in all these experiments is very small. While fetal brain brings about an appreciably more rapid deamination than glioblastoma, the activity of both tissues is negligible when compared to the rate of deamination of 8-aza-guanine in homogenates of non-neoplastic adult brain. The virtual absence of any azaguanine deaminase activity from homogenates of glioblastoma (cf. Table 2) is thus confirmed by the results obtained with the much more sensitive chromatographic procedure.

**DISCUSSION**

These findings support and extend the conclusions reached previously on the significance of azaguanine deaminase for the response of animal tumors to the drug (3). In the present instance, a human tumor has been shown to be severely damaged by 8-aza-guanine, but not at all by 8-aza-xanthine, in tissue culture; this tissue is unable to inactivate the carcinostatic agent at an appreciable rate. Fetal brain, which is somewhat less severely affected by 8-aza-guanine in tissue culture, is somewhat better able to carry out this inactivation. Adult human brain, which could not be tested in tissue culture, exhibits the highest azaguanine deaminase activity of any animal tissue studied so far, and may be expected to be relatively immune to damage by this agent.

This set of circumstances may appear to be very favorable to the clinical application of this drug to the treatment of human glioblastoma multiforme. However, it must be pointed out that the absence of azaguanine deaminase activity from this tissue is not the only, and probably not the main, factor which controls the level of the drug which can reach the tumor cells in vivo. The high rate of deamination of 8-aza-guanine in non-neoplastic adult brain and the probable extensive deamination of the agent by other normal tissues and body fluids (cf. 1, 3) may be expected to decrease the circulating level of the drug to a significant degree. Experiments are now in progress to assess this circulating level of 8-aza-guanine in patients treated with this drug on an experimental basis.

It should also be stressed that the damaging effects of 8-aza-guanine on glioblastoma cells have until now been demonstrated only in tissue culture, where the manifold complicating systemic factors do not come into play.

Despite these reservations, it is felt that the close correlation between the degree of damage of a tissue by 8-aza-guanine and the rate of inactivation of the drug by the cells of this tissue provides a suitable starting point for approaches to a more efficient use of this agent in experimental cancer chemotherapy.

**SUMMARY**

1. The human brain tumor, glioblastoma multiforme, when grown in tissue culture, is severely damaged by exposure to 8-aza-guanine.
2. Fetal human brain, the only available non-neoplastic control for tissue culture experiments, is less severely damaged by exposure to this agent and recovers more rapidly and more completely when the agent is removed.
3. 8-Aza-xanthine, the end product of the enzymatic deamination of 8-aza-guanine, causes no damage of any kind to either of these tissues in tissue culture.
4. Homogenates of glioblastoma are devoid of measurable azaguanine deaminase activity; homogenates of non-neoplastic adult brain exhibit the highest deaminase activity of any animal tissue which has been studied.
5. Chromatographic analysis of feeding fluids after incubation with glioblastoma explants in tissue culture confirms the virtual absence of azaguanine deaminase activity from the cells of this tissue. Fetal brain is somewhat better able to carry out the deamination of this agent, but even this slightly greater deaminase activity is very small when compared to the activity of adult human brain.

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Enzymatic Deamination of 8-Azaguanine in Normal Human Brain and in Glioblastoma Multiforme

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