Studies on the Effect of 8-Azaguanine on Sarcoma 37 in Mice

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Since the observations of Kidder et al. (8) that 8-azaguanine selectively inhibits the development of three types of mouse neoplasms, many investigators have examined the carcinostatic power of this compound on a variety of cancer tissues in different animal species. It has been found that 8-azaguanine is active against many additional malignant neoplasms (5, 6, 9, 10, 14, 16) but produces little or no effect on others (3, 5, 6, 9, 10, 15, 16). Some investigators (5, 8) have reported a lack of toxicity in the host at doses that inhibit tumor growth; others (6, 16) have not observed a well defined separation of the tumor inhibitory action of 8-azaguanine from systemic effects of the compound as manifested in reduced food intake, weight loss, leukopenia, morphological damage, and death. Few or insignificant histological changes in tumors inhibited by 8-azaguanine have been reported. Kidder et al. (8) reported that it did not cause the death of E 0771 cells in C57 mice but only checked their growth. Sugiuira and his associates (16), who also examined the effect of 8-azaguanine on E 0771 in C57 strain mice, observed small islands of active tumor tissue surrounded by gelatinous edema and necrosis. The most striking tumor damage was reported by Gellhorn et al. (5) and by Shapiro and his associates (14), who examined the histological changes induced in the Brown-Pearce carcinoma in rabbits. They observed that the tumor cells of treated rabbits were larger and fewer in number (5, 14), contained condensations of nuclear material in irregular clumps (5), and that the mitotic index was markedly reduced (14). While Shapiro et al. (14) also demonstrated that the mitotic indices of the intestinal epithelia and of the testes of treated rabbits were not affected, indicating a possible selective action of the drug, this inhibition of tumor growth was not completely dissociated from toxicity in the host, inasmuch as there was an appreciable loss in body weight. There were no evident cytological changes in other malignant neoplasms (5).

The present study involves an exploratory examination of the growth and morphological changes of Sarcoma 37 in CAF1 mice treated with 8-azaguanine and the effects of this compound on the host.

MATERIALS AND METHODS

Aseptic mashies of the rapidly growing Sarcoma 37, prepared according to the method described by Leiter et al. (11), were implanted into the right thigh muscles of CAF1 strain mice, 15–22 gm. in weight. For each experimental group there was a comparable group of untreated control mice bearing implants of the same tumor generation. Tumor volumes were estimated by caliper measurements in three dimensions and are reported here as the average increase in size expressed in cubic centimeters. The effect of the drug on tumor growth was graded as follows: (+ +), (+), (±) and (−) which corresponds, respectively, to an increase in size of 1/2 (marked inhibition), from 1/2 to 1 (moderate inhibition), from 1/2 to 1 (slight inhibition) and 1 or more (no effect) the increase in size of controls, respectively. Variations in concentrations of injected tumor mash accounted for the variation in size of control tumors between experiments (Chart 1). Within any one experimental series the mash concentration injected was relatively constant. Every mouse was weighed daily, observed for signs of systemic toxicity, examined at autopsy at the termination of an experiment, and each tumor was routinely examined for gross changes. Representative tumors and viscera were fixed in 10 per cent formalin or Zenker-formol solution; sections were stained with hematoxylin and eosin and evaluated histologically.

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The mice received Purina laboratory pellets and water ad libitum.

Tumor age at the time of initiation of prolonged therapy varied from 0 to 5 days; in mice that received a single dose, the tumors were 5–7 days old. At this time they showed little or no necrosis, except for the original implant. While necrosis occurs spontaneously in Sarcoma 37, this should not be confused with induced necrosis that was observed in mice undergoing 8-azaguanine therapy.

The 8-azaguanine was synthesized, with modifications, according to the method of Roblin et al. (13). Analysis by counter-current distribution indicated more than 95 per cent purity, and the ultraviolet absorption spectrum was identical with that described by Cavalieri et al. (4). Stock solutions were made in 0.25 N (or less) NaOH, prepared freshly at least once each week. Required dilutions of the drug were made in distilled water, so that the dose for injection was contained in 0.02 cc. per gram of body weight (volume equivalent to 2 per cent total body weight); this dose was administered subcutaneously into the axilla contralateral to the tumor implant.

In several experiments, control mice were divided into two groups. One group received NaOH of the proper dilution subcutaneously, while the other received none. There was no essential difference between these groups, and the results were, therefore, combined. Similarly, sex did not influence the effect of the drug on the tumor or the host; consequently, the results obtained with these two groups were also combined.

RESULTS

Effect on tumor growth.—The average results from 195 mice undergoing 8-azaguanine therapy at different dose levels in which drug administration was initiated at different time intervals after tumor transplant are summarized in Chart 1 and Table 1. It can be seen that at certain dose levels of 8-azaguanine there was relatively significant tumor inhibition. The tumor, however, was not destroyed or completely inhibited, inasmuch as there was progressive but very slow growth. Moreover, following termination of treatment with a dose of 175 mg/kg/day, tumors in mice that were not sacrificed grew rapidly and approached the size of controls. This observation is in accord with the findings of other investigators (5, 8, 16), who demonstrated growth inhibition of different malignant neoplasms.

At a dose of 100 mg/kg/day, the effect was no greater than "slight inhibition" regardless of whether therapy was initiated on the day of tumor transplant or up to 5 days later. Nevertheless, this effect in experiments 2 and 4 was statistically significant (probability < 0.01). Doses of 150 and 175 mg/kg/day produced statistically highly significant "moderate inhibition." It is interesting to note that the total doses (600–700 mg/kg) administered at this dose level (150 and 175 mg/kg/day).
day) were similar to the total doses (400-700 mg/kg) administered at the lower level (100 mg/kg/day) but that the former was more effective. Furthermore, even though the lower level therapy was initiated on the day of tumor transplant, as compared to the later initiation of therapy at the higher dose level, the effect was only relatively slight.

Effect on host.—Mortality due to drug toxicity was comparatively low, as indicated in Chart 1.

Table 1 indicates that there was a loss in body weight, during the period of therapy, in both control and treated mice. This observation was closely associated with anorexia that developed in mice that received daily subcutaneous injections of NaOH. There was essentially no change in weight in untreated control animals (Table 1, exp. 6).

Anorexia and loss in body weight were apparently due in part to NaOH injections. This observation was made in both normal and tumor-bearing mice, as is demonstrated in Chart 2. During the 24-hour period following a single subcutaneous injection of 500 mg/kg of 8-azaguanine in NaOH, or NaOH alone, there was marked anorexia in both normal and tumor-bearing mice. Intraperitoneal injections of a similar dose of the drug in water caused but slight anorexia. Essentially complete recovery was evident within 48 hours; however, the weight loss (up to 19 per cent) of both normal and tumor-bearing mice that received 8-azaguanine in NaOH was comparable to mice deprived of food for 48 hours and water for 24 hours. On the second post-injection day, mice that received the drug intraperitoneally in water showed a lesser degree of weight loss (approximately 10 per cent), comparable to the NaOH-treated controls. The slight loss in weight observed in some untreated controls may possibly have been due to environmental conditions in the laboratory.

There were no signs of diarrhea or other toxic manifestations in this group of mice or in those on a protracted course of 8-azaguanine treatment.

In both the treated and control mice there was a gelatinous edema which was limited to the site of injection, and after multiple injections there often developed an ulceration. These effects, which were apparently due to the relatively high concentration of NaOH, regressed when therapy was terminated. Moreover, there was no apparent correlation between these effects and tumor damage caused by the drug.

The most striking gross effect of repeated doses of the drug on the host was a diminution in the size of the spleen of both normal and tumor-bearing mice, which amounted to approximately 50 per cent of the size of control spleens (Table 1). This decrease in spleen size was seldom seen after a single dose of 500 mg/kg. In addition, there was a pronounced discoloration of the spleen on repeated
No gross changes were apparent in the stomach, intestines, pancreas, mesentery, kidneys, lung, liver, heart, or brain of 8-azaguanine-treated mice.

**Morphological tumor changes.**—Gross tumor changes were prominent in treated mice. Doses of 150 and 175 mg/kg/day produced a distinct diffuse pink color throughout the tumor mass, in contrast to the light color of the controls. The color was less in those mice that received 100 mg/kg/day. After doses of 175 mg/kg/day, there were also necrotic areas which were not grossly apparent in the controls of this group. Gross tumor damage was seen within 2–6 hours after the most striking damage observed 24 hours after a single dose of 500 mg/kg. This was similar in appearance to that seen after a single effective dose of podophyllotoxin (11). The tumors were soft and diffusely hemorrhagic and often appeared completely hemorrhagic except for occasional islands of apparently unaffected tissue. This damage was apparently reversible, inasmuch as progressively less damage was observed on subsequent days.

The gross tumor damage and the inhibition of tumor growth by 8-azaguanine were closely associated with marked histological changes in the tumor. Examination of a tumor excised 24 hours after a single dose of 500 mg/kg (Figs. 1 and 2) revealed relatively few undamaged cells, but most of the tumor showed marked vascular damage, hemorrhage, and stasis along with pronounced cellular degeneration. Cytoplasmic damage was evidenced by eosinophilia, vacuolization, retraction, and disintegration. The changes observed in the nuclei were of several types: (a) pyknotic, clumped chromatin granules; (b) swollen, often fading nuclei, with disorganization or loss of distinct granules; (c) karyorrhexis, chromatin dust, nuclear debris; and (d) distortion of most of the visible mitoses. These cellular alterations are interpreted as representing various stages and possibly various types of degenerative processes. The mitotic figures included all stages, but were approximately one-fifth as frequent as in control tumors (Table 2). The number of cells was also reduced.

Forty-eight hours after a single dose of 8-azaguanine, cellular disintegration remained evident. However, the relative number of mitotic figures in viable areas had nearly returned to that of control tumors, thus paralleling the gross observations and indicating reversibility of 8-azaguanine action.

At the time of sacrifice, 24 hours after the last daily dose of 175 mg/kg/day, the tumors were of the same age as those described above, but were appreciably smaller. Histologically, they showed numerous necrotic foci, little hemorrhage, few distorted mitotic figures, and the mitotic number approached that of control tumors.

Tumors of mice that received daily doses of 100 mg/kg, which showed no appreciable inhibition in growth, revealed histological damage comparable to that in those mice which received the higher daily dose of the drug.

Tumor inhibition was not the result of malnutrition, since mice deprived of food and water showed a weight loss comparable to that seen after 8-azaguanine (Chart 2), but this was not accompanied by retarded tumor growth or morphological evidence of tumor damage.

**Histological changes in the host.**—No histological changes were observed in the spleen and the adrenal glands after a single dose of 500 mg/kg of 8-azaguanine. It will be recalled that spleens of mice that were treated daily were much smaller than control spleens, usually brown in color, and often translucent. A number of the adrenals were similarly discolored. On microscopic examination, the sinusoids in the spleen were not distinct, as if compressed, and there were comparatively few erythrocytes present. The cortex and medulla of the adrenal glands showed cytoplasmic vacuolization, and the inner zone of the fasciculata possessed large areas of degenerative cells. Similar

**TABLE 2**  
**The Effect of a Single Dose of 8-Azaguanine on the Mitotic Rate of Sarcoma 87 in Mice***

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Cells Mitoses/Per cent</th>
<th>Control</th>
<th>8-Azaguanine</th>
</tr>
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<tr>
<td></td>
<td>hpf</td>
<td>Mitoses</td>
<td>Per cent</td>
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<tr>
<td>1</td>
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<td>9</td>
<td>1.14</td>
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<td>785</td>
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<td>0.70</td>
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<td>4</td>
<td>785</td>
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<td>8</td>
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<td></td>
<td>Mean 768</td>
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<tr>
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<td>Mean 765</td>
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</table>

* Tumor removed 48 hrs after a single dose of 500 mg/kg; areas counted were chosen at random.
† hpf = high-power field.
changes in the spleen and adrenal glands were observed in treated nontumor-bearing mice.

Representative tumors and spleens of mice that received the multiple injections of 8-azaguanine were employed in respiration studies in the Warburg apparatus and are reported in the following paper.

DISCUSSION

Inhibition of growth of Sarcoma 37 was observed after treatment with 8-azaguanine, in association with tumor damage and gross and histological signs of systemic toxicity. The discrepancy between these results and those of Greenberg et al. (7) and of Kidder et al. (9), who stated that they observed no response of this tumor to 8-azaguanine, is probably explained by a difference in dose level and/or a difference in the route of administration. Although an appreciable inhibition in growth with the lowest dose employed in this investigation (100 mg/kg) was not always observed, induced histological changes were evident.

A well defined separation of the tumor-inhibitory action of 8-azaguanine from the systemic effects of the drug was not apparent, and there was no appreciable difference between treated normal and tumor-bearing mice. These observations seem to indicate that the effects of the drug may result from a specific cytotoxic drug action and not necessarily from an exacerbation of a toxic or alarm reaction activated by the tumor, or that there may be a combination of a specific cytotoxic effect and an alarm reaction induced by 8-azaguanine. In experiments reported here it was observed that the administration of this drug caused anorexia, weight loss, and morphological changes in the spleen and adrenal gland, but other gross changes in vital organs were not observed. Sugiura et al. (16) observed extreme congestion and fine granules in the liver cytoplasm, extensive congestion in the kidneys, and a reduction of lymphoid tissue in the lymph nodes. Goldin et al. (6) reported that it produced systemic toxicity as seen in reduced food intake, weight loss, leukopenia, and death.

The gross observations suggest that there is no apparent accumulation of the drug in the body and that multiple injections of low doses do not produce a progressively increased gross effect on Sarcoma 37. The former observation is supported by the findings of Bennett et al. (2), who reported that approximately 95 per cent of the activity of injected 8-azaguanine-2-C\(^{14}\) was eliminated within 24 hours. Moreover, Mandel et al. (1) observed that approximately 50 per cent of the activity of injected 8-azaguanine-4-C\(^{14}\) was eliminated within 2 hours and approximately 98 per cent within 12 hours. It may be assumed, therefore, that a substantially effective dose must be employed from the outset in order to elicit a significant inhibition of growth of Sarcoma 37. This may be further supported by experiment 4b (Chart 1), in which an initial dose of 500 mg/kg was followed by daily doses of 100 mg/kg. This form of "vigorous" therapy produced "marked inhibition."

The observations that 8-azaguanine produced extensive hemorrhage and stasis in the tumor mass may indicate that the necrosis is caused by an interference with the vascular supply, as has been proposed by Ludford (12) and by Algire et al. (1) for the mode of action of colchicine and bacterial polysaccharides. However, this does not appear to be a satisfactory explanation of the action of this compound, since other authors have not reported vascular degeneration or hemorrhage in tumors inhibited by 8-azaguanine, in those that showed induced necrosis, or in lymph nodes that were affected by this drug (10). Furthermore, in the present investigation, hemorrhage in spleens or adrenal glands, or an obstructed blood supply in the necrotic adrenals, was not observed.

SUMMARY

1. Data are presented on the effect of single and multiple doses of 8-azaguanine on growth, morphology, and drug toxicity in normal and tumor-bearing mice.

2. Multiple doses of 8-azaguanine produced a definite inhibition of growth and extreme cellular damage of Sarcoma 37.

3. Gross tumor damage was observed within 2–6 hours, with extensive morphological damage 24 hours after a single large dose of 8-azaguanine.

4. The number of mitotic figures in tumors after repeated doses of 8-azaguanine approached that of control tumors, whereas after a single large dose it was approximately one-fifth that seen in controls.

5. The drug was not completely destructive to this tumor. Progressively less effect on growth and morphology was seen on cessation of therapy.

6. No well defined separation of the tumor-inhibitory action of 8-azaguanine from systemic and morphological effects of the drug was apparent. The toxic action was similar in normal and tumor-bearing mice. Multiple doses produced morphological spleen and adrenal damage.

REFERENCES


In press.


Fig. 1.—Sarcoma 37, untreated.

Fig. 2.—Sarcoma 37, 24 hours after a single dose of 500 mg/kg of 8-asaguanine.
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