Inhibition of Cell Proliferation by Azathioprine

Daniel Malamud, Eduardo M. Gonzalez, Hua-i Chiu, and Ronald A. Malt

Surgical Services, Massachusetts General Hospital, and the Shriners Burns Institute, Boston 02114, and Department of Surgery, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

The rise in DNA synthesis in regenerating rat liver is inhibited by azathioprine (40 mg/kg). Regeneration in the presence of azathioprine occurs by hypertrophy as assessed both by an increase in the ratio of RNA or protein to DNA and by an increase in diameter of the hepatocytes. Mitoses that occur in azathioprine-treated regenerating liver result from a G2 population that is apparently stimulated by the drug. Azathioprine also inhibits DNA synthesis in normal liver, spleen, kidney, duodenum, thymus, and lymph nodes.

INTRODUCTION

Although azathioprine (Imuran) was originally synthesized to serve as a depot for the slow release of 6-MP for cancer chemotherapy (13, 14), its principal use has been as an immunosuppressive (3) and antiinflammatory agent (1). Since the 6-MP moiety released from azathioprine probably interferes with both the de novo synthesis of purines and the interconversion of purine nucleotides (7, 17), potential effects of azathioprine on the synthesis of both DNA and RNA might be expected. In addition to these actions, azathioprine can be converted directly to 6-thiouric acid without the release of 6-MP (4), thereby perhaps allowing a mechanism of action unique to azathioprine.

Previous studies have shown that azathioprine (10) and 6-MP (15) inhibit DNA synthesis in regenerating rat liver. In the presence of azathioprine, restoration of liver mass appears to occur by hypertrophy rather than by hyperplasia. Studies to be described in this paper demonstrate the differential effects of azathioprine on DNA and RNA synthesis in regenerating liver and in other organs and substantiate the fact that hepatic regeneration in the presence of azathioprine occurs largely by hypertrophy.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Gofmoor Farms, Westboro, Mass.; 250 g) were housed in individual cages and were pair fed, beginning 2 days before 68% hepatectomy (11). Azathioprine4 was administered by p.o. intubation at a dosage of 40 mg/kg in a suspension of 1% carboxymethylcellulose on the night before hepatectomy, 2 hr before the operation, and at 24 hr intervals after hepatectomy. Control rats received 1% carboxymethyl-cellulose at the same intervals.

For comparative studies of DNA and RNA synthesis, male Charles River mice (30 g) were given a single injection of the sodium salt of azathioprine (40 mg/kg). Control mice were injected with 0.9% NaCl solution adjusted to the same pH (about 9.8).

For evaluation of DNA synthesis in rats, animals were injected with thymidine-methyl-3H (6.7 Ci/m mole) at dosages and times indicated in the figure legends. Mice were given injections of a mixture of 10 μCi of thymidine-methyl-14C (54.7 mCi/m mole) and 50 μCi of uridine-5-3H (15.9 Ci/m mole), and were killed after 45 min. RNA and DNA fractions were prepared as described by Scott et al. (19), modified by Hinrichs et al. (12).

Autoradiographic studies were carried out with the use of Kodak NTB-2 emulsion and with an exposure time of 2 to 4 weeks (2). Slides were stained through the emulsion with hematoxylin and eosin.

RESULTS

Twenty-four hr after partial hepatectomy alone, autoradiography revealed a 10-fold increase in the fraction of cells labeled with thymidine-3H. This stimulation of DNA synthesis was completely inhibited in the regenerating liver of rats treated with azathioprine (Chart 1).

With the exception of the bile duct epithelium, the extent of inhibition of DNA synthesis was equal in the several cell types studied (Table 1). The inhibition of DNA synthesis was due to a decrease in the number of cells synthesizing DNA, rather than to a decreased rate of DNA synthesis per cell, since there was little change in the number of grains per cell in azathioprine-treated animals.

Because DNA synthesis in regenerating liver of azathioprine-treated rats was inhibited 80 to 85%, whether measured by DNA-specific activity or by the labeling index following thymidine-3H injection, it was anticipated that the mitotic index would be similarly depressed. In fact, although there was a complete inhibition of mitosis at Hr 24 posthepatectomy, there was a modest increase in the mitotic index at Hr 48 after hepatectomy in azathioprine-treated animals (Chart 2). This increased mitotic index in the absence of DNA synthesis suggested the existence of a G2 population.

Two experiments were carried out to investigate this
possibility (Table 2). In the 1st, rats were injected with thymidine-\( ^3 \)H at 5-hr intervals for 15 hr preceding the 48-hr time point. This labeling, which should have been sufficient to label all mitotic cells undergoing a normal G2 period (8), revealed an increase in the percentage of unlabeled mitotic figures in azathioprine-treated rats. When rats were exposed to thymidine-\( ^3 \)H at 5-hr intervals for the entire 48 hr, there still was an increase in the percentage of unlabeled mitoses in the drug-treated group as compared with rats that were hepatectomized only.

Azathioprine also markedly inhibited DNA synthesis in rat spleen, lymph node, and duodenum (Chart 3). Since there was relatively little incorporation of thymidine-\( ^3 \)H into control thymus, it was difficult to assess the effect of azathioprine on this tissue.

To check the previous suggestion that hepatic regeneration in the presence of azathioprine occurred by hypertrophy rather than hyperplasia (10), we determined the cell diameter and the number of cells per high-power field (Table 3). There was an increase in cell diameter after treatment with azathioprine and a comparable decrease in the number of cells per unit area. These changes were of the same order of magnitude as the biochemical results on RNA/DNA and protein/DNA.

![Chart 1](image)

**Chart 1.** Effect of azathioprine on DNA synthesis in regenerating liver. Rats were hepatectomized and given azathioprine or vehicle, as described. Thymidine-methyl-\( ^3 \)H (100 µCi) was injected i.p. at the indicated times, and the rats were killed 1 hr later. Slides were dipped in NTB-2 (nuclear track emulsion) and exposed for 3 weeks. At least 1000 cells from each rat were counted. ●, hepatectomy only; ○, hepatectomy plus azathioprine.

![Chart 2](image)

**Chart 2.** Effect of azathioprine on mitosis in regenerating liver. Partially hepatectomized rats were given azathioprine or vehicle only, as described in text. At the time intervals indicated, rats were killed and tissue sections of liver were prepared. Each point on the graph represents an analysis of at least 1000 hepatocytes. ●, hepatectomy only; ○, hepatectomy plus azathioprine.

### Table 2

**Effect of azathioprine on the percentage of unlabeled mitoses**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>15 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatectomy only</td>
<td>1.3%</td>
<td>2%</td>
</tr>
<tr>
<td>Hepatectomy + azathioprine</td>
<td>38.4%</td>
<td>19.4%</td>
</tr>
</tbody>
</table>

* No. in parentheses, actual values for each rat.

### Table 1

**Effect of azathioprine on hepatic DNA synthesis**

Rats were hepatectomized and given azathioprine or vehicle p.o., as described in text. Thymidine-methyl-\( ^3 \)H (30 µCi) was injected at Hr 2 after hepatectomy and at 5-hr intervals until rats were killed at Hr 48 after hepatectomy. The DNA-specific activity of a piece of liver tissue was determined. A 2nd piece of tissue was used for autoradiographic preparations. Approximately 200 cells were counted for each determination of the labeling index and 30 cells for grain counting. There were 4 rats in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatocyte</th>
<th>Blood vessel</th>
<th>Capsule</th>
<th>Bile duct</th>
<th>Hepatocyte grain count</th>
<th>DNA-specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatectomy only</td>
<td>662</td>
<td>553</td>
<td>700</td>
<td>723</td>
<td>28.7</td>
<td>482</td>
</tr>
<tr>
<td>Hepatectomy + azathioprine</td>
<td>119</td>
<td>133</td>
<td>130</td>
<td>373</td>
<td>21.3</td>
<td>75</td>
</tr>
</tbody>
</table>

* No. of labeled cells per 1000 total cells.

### Table 3

**Effect of azathioprine on the number of cells per high-power field**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatocyte</th>
<th>Blood vessel</th>
<th>Capsule</th>
<th>Bile duct</th>
<th>Hepatocyte grain count</th>
<th>DNA-specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatectomy only</td>
<td>18</td>
<td>24</td>
<td>19</td>
<td>52</td>
<td>74</td>
<td>15</td>
</tr>
</tbody>
</table>

* Average no. of grains over hepatocytes.

DNA-specific activity expressed as dpm/mg DNA \( \times 10^{-3} \).
Chart 3. Inhibition of DNA synthesis by azathioprine. Rats were given daily intubations of azathioprine, or of vehicle only, for 2 or 3 days. Thymidine-methyl-3H (50 μCi) was injected i.p. at Hr 15, 10, and 5 before the rats were killed. Results are expressed as DNA-specific activity x 10^-3. Percentages over bars, percentage of inhibition of DNA synthesis.

Table 3
Effect of azathioprine on cellular hypertrophy

Rats were studied 4 days after hepatectomy only or after hepatectomy plus daily p.o. administration of azathioprine. RNA/DNA and protein/DNA were determined, as previously described. Cell diameter and number of cells per high-power field were determined on 10-μm tissue sections.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RNA/DNA</th>
<th>Protein/DNA</th>
<th>Cell diameter (μm)</th>
<th>No. of cells/high-power field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatectomy only</td>
<td>4.36 ± 0.18a</td>
<td>77.02 ± 1.71</td>
<td>21.4 ± 0.87</td>
<td>39.0 ± 2.03</td>
</tr>
<tr>
<td>Hepatectomy + azathioprine</td>
<td>6.95 ± 0.70</td>
<td>103.40 ± 9.68</td>
<td>28.8 ± 1.34</td>
<td>27.3 ± 2.49</td>
</tr>
<tr>
<td>% change</td>
<td>+59</td>
<td>+34</td>
<td>+35</td>
<td>-31</td>
</tr>
</tbody>
</table>

* Values represent mean ± S.E. for 15 rats.

In the experiments with mice performed to determine the effect of a single injection of azathioprine on DNA and RNA synthesis, nucleic acid synthesis was depressed at 48 hr after a single injection of the drug (Table 4). In general, DNA synthesis was inhibited to a greater extent than was RNA synthesis.

**DISCUSSION**

Azathioprine (40 mg/kg, ~210 mg/sq m) thus effectively suppresses the stimulation of DNA synthesis after partial hepatectomy. This effect is seen in all cell types studied and represents an inhibition of the number of cells synthesizing DNA, rather than a decrease in the rate of DNA synthesis per cell.

Although the mitotic index is also depressed in regenerating livers of azathioprine-treated rats, there is a modest stimulation of mitoses at Hr 48 after hepatectomy, and these represent (at least in part) a G2 population since, after multiple thymidine-3H injections, about 20% of the mitoses were unlabeled, as compared with only 2% in the absence of azathioprine. Immunosuppression has previously been reported to stimulate proliferation of a G2 population a few hr after injection of either hydrocortisone or antilymphocyte serum (5, 6).

Despite a priori considerations suggesting that purine
analogs might depress the synthesis of both DNA and RNA, the present findings suggest that azathioprine specifically inhibits DNA synthesis, even after a single injection of the drug. As previously noted (10), liver weight and total RNA increase following heptectomy in the presence of azathioprine so that growth occurs by hypertrophy rather than hyperplasia. This finding is further substantiated in the measurements showing that, by RNA/DNA, protein/DNA, cell size, or by number of cells per high-power field, azathioprine-treated regenerating liver undergoes hypertrophy, compared with liver regenerating in the absence of the drug. A relatively greater inhibition of DNA synthesis, compared with RNA synthesis, has been reported by Salser et al. (18) for 6-MP and is suggested by the data of Glen et al. (9) for azathioprine. Although we cannot explain why a purine analog should specifically affect DNA synthesis, this would not appear to be consistent with an inhibition of an enzyme in the synthetic pathway for purines.

It has also been found that inhibition of DNA synthesis in regenerating rat liver by azathioprine is reversible. When drug administration is stopped at Day 4 after partial hepatectomy, there is a marked increase in DNA synthesis and a concomitant decrease in RNA/DNA (T. Van Vroonhoven, D. Malamud, and R. A. Malt, manuscript submitted). After cortisol administration (16), a remarkably similar sequence of events occurs in which there is a reversible inhibition of cell proliferation in regenerating liver after steroid administration. Thus, 2 immunosuppressant agents appear to exert a similar antiproliferative effect on regenerating liver.

ACKNOWLEDGMENTS

We thank Dr. Robert A. Hershberg for his help with the histological analysis.

REFERENCES

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