Azathioprine Induction of Lymphomas and Squamous Cell Carcinomas in Rats

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ABSTRACT

The carcinogenicity of azathioprine was evaluated in weanling female noninbred Sprague-Dawley rats by feeding it in the diet. Due to toxicity, the dose had to be changed during the course of the experiment and ranged from 0.015 to 0.04% of the diet by weight. In the first experiment, the estimated maximal cumulative consumption of azathioprine was 1.5 g/rat. Of the 14 rats evaluated, six developed thymic lymphomas, and four developed squamous cell carcinomas of the ear duct. When the experiment was repeated with a slightly lower daily consumption but with a cumulative total dose of 2.2 g/rat, there were seven of 19 rats with thymic lymphoma and two rats with ear duct carcinoma. These data support the hypothesis that azathioprine is a carcinogen.

INTRODUCTION

Azathioprine, an immunosuppressant analogue of 6-mercaptopurine, is used clinically in patients undergoing organ transplantation and in the treatment of a variety of autoimmune diseases (16, 19, 23). Its use in transplant and in nontransplant patients has been related to the subsequent appearance of a high frequency of lymphomas, particularly those of B-cell origin, and also with frequent squamous cell carcinomas at various sites, particularly skin and cervix (19, 23). It remains unclear what the exact role of azathioprine is in the induction of these tumors since there are other variables (19), including use of other drugs, the presence of autoimmune disorders or an allograft, or additional immunosuppressive agents such as antithymocyte or antilymphocyte globulin. The role of radiation in some of these patients is also unclear. The age and sex of the patient and their geographical locale are also determinants.

In animals, azathioprine has not been adequately evaluated for carcinogenic activity (22, 23, 28). In mice, it appears to be leukemogenic, but this usually involves strains of mice with autoimmune disorders (2, 3, 12, 17, 18), such as NZB, or mice that have also been given an antigenic stimulus (10, 15). In rats, lymphomas have not been induced, but ear duct squamous cell carcinomas have been reported in low incidences in Fischer rats given low doses of azathioprine for prolonged periods of time (9). The rat has the advantage as an experimental animal for the evaluation of the carcinogenicity of azathioprine since it has a low to absent rate of spontaneous thymic lymphomas in contrast to mice in which these tumors frequently occur spontaneously.

In addition to the 6-mercaptopurine portion of the molecule, azathioprine has a nitroimidazole substituent attached to the purine portion by a thiol bridge (6, 7). As part of our evaluation of a variety of nitroaromatic chemicals for possible carcinogenic activity, azathioprine was evaluated.

MATERIALS AND METHODS

Azathioprine was obtained from Burroughs Wellcome and Co. (Tuckahoe, N. Y.) and mixed in powdered Wayne Lab-Blox (Allied Mills, Chicago, Ill.) at the levels described in Tables 1 to 3. Weanling female Sprague-Dawley rats (Sprague-Dawley, Inc., Madison, Wis.) weighing 40 to 70 g at the start of the experiment were used. The rats were housed 4 per cage, and the number of rats in each group is shown in the tables. Control groups consisted of rats fed the powdered diet without the added azathioprine. Rats were weighed, food and chemical consumptions were determined periodically, and rats were palpated for tumors biweekly beginning at the sixth week. No antibiotics, other drugs, or food supplements were administered. When a rat died or was killed at the end of the experiment, a complete autopsy was performed. Tissues routinely fixed and processed for histopathological examination included lungs, thymus, liver, spleen, kidneys, urinary bladder, and breasts, and any other grossly abnormal appearing tissues. Tissues were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for histopathological evaluation. The incidences of tumors were based on the number of rats alive in each group after 12 weeks. Animals dying before that time were not included in the incidences. Statistical comparisons of tumor incidences of chemically treated rats with those in the appropriate unmedicated controls were calculated by the exact method for 2 × 2 tables (26).

In a preliminary experiment to evaluate toxicity, the dose schedule shown in Table 1 was administered. These doses were found to be severely toxic, resulting in the death of most of the animals early in the experiment with only 5 rats surviving through Day 67 of the experiment. Most of these rats died with respiratory distress, occasionally with hydrothorax. Those surviving for greater than 2 weeks frequently had the appearance of bronchopneumonia. Based on the results of this preliminary toxicity study, an experiment was performed to evaluate the long-term effects of azathioprine in the diet.

Fifty-six rats were divided into 2 groups as shown in Table 2 with 36 rats receiving azathioprine at an initial dose of 0.04% of the diet. Twenty rats were fed only the powdered control diet. This initial dose proved to be quite toxic, inhibiting growth and killing several of the rats. The dose was lowered, and the eventual schedule administered during the experiment is shown in Table 2. The effective number of rats in the experiment includes those that survived 10 weeks or more of the experiment.

Based on the results in this experiment, it was repeated beginning with the dose of 0.03% of azathioprine in the diet, and again, the dose had to be lowered due to toxicity. In this experiment (Table 3), 28 rats were present in the group administered azathioprine initially, and 19 survived 10 weeks or more. Thirty rats were present in the unmedicated control group.

RESULTS

Experiment 1. The cumulative consumption of azathioprine was 1.5 g/rat/total period of administration. This is a maximum
Azathioprine Carcinogenesis

estimate since it does not take into account diet spillage. The growth of rats receiving azathioprine was markedly retarded compared to the control animals (Chart 1). There was some resumption of growth during the periods of time when the azathioprine was removed from the diet, but the animals never attained the same weight as did the control rats. The types and incidences of the tumors induced by azathioprine are shown in Table 2. The lymphomas always involved marked enlargement of the thymus and in all but one case also involved marked enlargement of the spleen and multiple lymph nodes in the chest and abdomen as well as the cervical region. Infiltration of liver and kidneys was occasionally seen. The thymus gland nearly filled the thoracic cavity and histologically showed a diffuse proliferation of small lymphoid cells (Figs. 1 and 2), typical of thymic lymphomas as seen in mice (2, 3, 15, 17) and rats (1). The histological pictures in the lymph nodes and spleen were similar to that in the thymus. Rats with lymphoma died during Weeks 26, 35, 36, 47, and 66 of the experiment. The tumors of the ear duct and skin (most likely of Zymbal's gland origin) were ulcerating. Histologically, they were well-differentiated, keratinizing, squamous cell carcinomas invading the underlying connective tissue (Fig. 3). In one case, there was invasion of the underlying skeletal muscle and bone. In the tumors of the ear duct, there were features suggestive of sebaceous differentiation focally (Fig. 4). Ear duct tumors were first detached in rats during Weeks 42, 50, 55, and 66 of the experiment. The rat with the liver tumors had multiple tumor nodules with areas of hepatoma, cholangiocarcinoma, and angiosarcoma. The breast tumors were classified according to criteria described previously (8). In addition to these tumors, 2 rats showed moderate transitional cell

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<tr>
<th>Table 1</th>
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<td>Group</td>
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<td>Azathioprine</td>
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| Chart 1. Growth of rats fed azathioprine compared to rats fed only control diet (Experiment 1). |

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<th>Table 2</th>
<th>Carcinogenicity of azathioprine (Experiment 1)</th>
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<td>Group</td>
<td>Dose (%)</td>
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* Compared to control group, p < 0.01.  
* Compared to control group, p < 0.05.

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<th>Table 3</th>
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<td>Group</td>
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* Compared to control group, p < 0.001.  
* Compared to control group, p > 0.1.
hyperplasia of the renal pelvis, and one rat showed mild hyperplasia of the urothelium of the urinary bladder. One rat showed severe damage to the liver involving liver necrosis, a lymphocytic infiltrate, bile duct proliferation, and fibrosis. Of the 14 rats fed azathioprine which survived greater than 12 weeks, 10 of the rats had tumors, and 4 of the rats had no evidence of tumor. In the control rats, 2 had fibroadenomas of the breast.

**Experiment 2.** The total chemical consumption of azathioprine was 2.2 g/rat/total time of administration. Again, there was growth retardation in the rats given azathioprine as illustrated in Chart 2. The incidence of tumors is listed in Table 3. The histological analyses of the lymphoma and squamous cell carcinomas and breast tumors were the same as in the previous experiment. Rats with lymphoma died during Weeks 24, 26, 31, 32, and 39, and the ear duct tumors were first detected during Weeks 29 and 52 of the experiment. Of the 19 rats given azathioprine that survived 12 weeks or more of the experiment, 7 developed lymphoma, and 2 developed squamous cell carcinoma. Ten rats fed azathioprine had no tumors present.

**DISCUSSION**

Azathioprine was shown to be carcinogenic in female Sprague-Dawley rats, inducing statistically significant incidences of thymic lymphomas and squamous cell carcinomas of the ear duct. The induction of thymic lymphomas in rats by azathioprine has not been reported previously (23). These lesions are morphologically similar to the thymic lymphomas that occur in mice both spontaneously and in response to various viruses or chemicals. Azathioprine has been associated with the induction of such tumors in mice, but frequently, this has been in association with chronic exogenous antigenic stimulation (15) or administration to mice having autoimmune disorders (2, 3, 12, 17, 18). Particularly in the latter cases, it is unclear whether azathioprine was actually inducing the lymphoma or prolonging the life of the mice by treating the autoimmune disorder and thereby allowing for time for the appearance of the lymphomas. In the present experiment, the longevity of the control rats was longer than those treated with azathioprine; lymphomas appeared considerably earlier than the actual termination of the experiment. Although the immunosuppressive activity of azathioprine was not measured in the present experiment, similar doses were immunosuppressive in Fischer F344 rats (13). The role of the severe toxicity observed in the present experiment in the genesis of the lymphomas and carcinomas is not clear. Especially during the early periods of each experiment, the dose appeared to be above the maximally tolerated dose.

The lymphomas related to azathioprine administration in rats and in mice appear to be of T-lymphocyte origin and may be related to the activation of RNA viruses, particularly in mice (1, 10, 12, 15, 16). In contrast, the lymphomas appearing in immunosuppressed humans are usually of B-lymphocyte origin and may be related to Epstein-Barr virus rather than RNA tumor viruses (11). The T-cell origin of the lymphomas in the present study, although probable, was not confirmed by immunohistochemical studies. Also, the potential role of viruses in the present experiment was not determined.

Rather than a single factor being responsible for the appearance of lymphomas, whether in animals or in humans, there is most likely an interaction between multiple variables, such as chemical, viral, and immunosuppression. Azathioprine may be acting by a variety of mechanisms in the induction of lymphomas. One possibility is that azathioprine is a complete carcinogen. In contrast, another mechanism would involve azathioprine interacting with the DNA of the lymphoid cells and simultaneously suppressing lymphocyte activity. This would then provide a mitogenic stimulus for the proliferation of lymphocytes (in the instance of the mouse and rat, apparently T-lymphocytes), which then gives rise to a second event which is critical for the appearance of these tumors. Lymphomas in humans secondary to immunosuppressive therapy with azathioprine include patients with continuous antigenic stimulation (transplant) and patients without transplantation (16, 19). Since exogenous antigenic stimulation is also not apparent in the present experiment in rats, it would appear that antigenic stimulation is not necessary for the evolution of the lymphomas. It does appear, however, that antigenic stimulation does increase the incidence of such lesions, since transplant patients develop lymphoma considerably more frequently than do nontransplant patients administered azathioprine (19), and similar results have been found in mice (10, 15).

The appearance of squamous cell carcinomas in the present experiment arising in the ear duct (probably originating in Zymbal's gland) does not directly correlate with any tumor in the human, but squamous cell carcinomas in skin and cervix are frequently seen in immunosuppressed patients receiving azathioprine (19). Interestingly, Zymbal's gland is a common site of tumor induction with aromatic amine and amide carcinogens in animals (9), suggesting that a common intermediate, the N-hydroxylamine, may be involved in the induction of these tumors by azathioprine as well as the aromatic amines and amides. Azathioprine has a nitroimidazole moiety (6, 7), and it has been shown that azathioprine is mutagenic in the Ames assay if anaerobic conditions are used (24, 27). This suggests that mutation in the Ames assay is due to reduction of the nitro group by the bacteria. Nitroimidazoles have been shown to undergo nitroreduction in vivo and in vitro with the formation of a hydroxylamine as the intermediate (14, 25). A few nitroimidazoles have been evaluated for carcinogenic activity, but in general, they have shown only weak tumorigenic activity (5, 20, 21). However, the nitroimidazoles that have been tested so far have been monocyclic and would not be anticipated to have strong carcinogenic activity, if any (4, 5).

In the evaluation by the International Agency for Research on Cancer of azathioprine (23), it was determined that it was a
carcinogen in humans but that the evidence was not sufficient for animal experiments. The experiment reported here provides support for the carcinogenicity of azathioprine in an animal model that does not involve exogenous antigenic stimulation or animals with autoimmune disease.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Malignant thymic lymphoma in a rat treated with azathioprine. H & E, × 80.
Fig. 2. Higher magnification of section shown in Fig. 1. H & E, × 200.
Fig. 3. Well-differentiated, invasive, keratinizing squamous cell carcinoma of the ear duct in a rat treated with azathioprine. H & E, × 80.
Fig. 4. Squamous cell carcinoma with areas suggestive of sebaceous differentiation. H & E, × 80.
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