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Induction of Uterine Hemangioendothelioma and Lymphoma in (C57BL/6N×C3H/2N)F1 Mice by Oral Administration of Azathioprine

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The tumorigenicity of 6-(1-methyl-4-nitro-5-imidazolyl)mercaptopurine (azathioprine), an immunosuppressant, was examined in (C57BL/6N×C3H/2N)F1 mice. Animals were divided into 6 groups with 50 mice in each group, and they were given powdered diet mixed with 0, 5 or 20 ppm azathioprine starting at 6 weeks and ending at 100 weeks of age. Female mice, given 20 ppm azathioprine, developed lymphomas and uterine hemangioendotheliomas at incidences of 12.5 and 14.6%, respectively, which were significantly higher than the incidences in control mice ($P<0.05$). Lymphoma and uterine hemangioendothelioma developed independently in different mice. On the other hand, the male mice given 20 ppm azathioprine had a significantly ($P<0.05$) lower incidence of hepatic tumor (0.5%) compared to the control mice (16%).

Key words: Azathioprine — Uterine hemangioendothelioma — Lymphoma — Mice

Because of the increase of organ transplantation for therapeutic purposes, there is a great need for immunosuppressive agents to prevent allograft rejection. However, the occurrence of certain types of cancer has been reported after immunosuppressive treatment.[1,2]

Azathioprine, a derivative of 6-mercaptopurine, as well as cyclosporin, is a potent immunosuppressant. Interaction of this chemical with cells induces cell death through damage to nucleic acid metabolism. Lymphocytes appear to be most susceptible to the deficiency of adenosine deaminase, loss of which causes severe impairment of both cellular and humoral immunity.[3,4] Although there have been studies on the carcinogenicity of azathioprine in rats,[5,6] experiments in mice have also been conducted, mostly with mice having autoimmune disease, and a high frequency of lymphomas was noted.[7,8]

In this study, a common hybrid strain of B6C3F1 mice was used, since this strain of mice has been extensively used for carcinogenicity studies.[9–12]

MATERIALS AND METHODS

Animals and chemical We obtained 150 male and 150 female weanling (C57BL/6N×C3H/2N)F1 (B6C3F1) mice from Charles River Japan Inc., Kanagawa. They were 4–5 weeks old and weighed 17.4 to 22.5 g (group averages) at the beginning of the experiment. Mice were housed 6 or 7 per cage in clean plastic cages and kept in a room with a controlled temperature of 24±2°C and humidity of 55±5%. All mice were given a basal diet (CRF-1; Oriental Co. Ltd., Tokyo) *ad libitum*. Azathioprine [6-(1-methyl-4-nitro-5-imidazolyl)mercaptopurine, Tanabe Pharmaceutical Co. Ltd., Tokyo] was administered orally at a dose of 0, 5, 20 or 100 ppm mixed in powdered diet starting at 6 weeks of age in the preliminary study. Since 100 ppm of azathioprine was highly toxic, doses of 0, 5 and 20 ppm of azathioprine were administered for 100 weeks without interruption.

Experimental design Mice were divided into the following groups. Group 1 (male) mice were given 20 ppm (group 1-a) or 5 ppm (group 1-b) azathioprine in their powdered diet. Group 2 (female) mice were given 20 ppm (group 2-a) or 5 ppm (group 2-b) azathioprine in their powdered diet. Control groups 1-c and 2-c were given normal powdered diet. Intake of azathioprine was also monitored.

Examination and pathological studies All mice were observed every day and weighed once a month. They were observed maximally for 100 weeks after azathioprine treatment. At 60 weeks and thereafter, sick animals with tumors were killed. At 86 and 94 weeks after azathioprine treatment (hereafter "weeks" indicate time after azathioprine treatment), about 10 mice in each of 4 experimental and 2 control groups were killed for the studies on the hematopoietic system, and remaining mice were killed for autopsy at 100 weeks. The body and individual organs were weighed and routinely processed for histological studies. The effective number of mice is the sum of animals that died or were killed after the first occurrence of neoplasm, a systemic lymphoma in a female mouse of group 2-a at 60 weeks.

At the time of death, imprints of thymus, liver and mesenteric lymph node involved in lymphoma were made on clean slide glasses and stained for anti-$Thy_{1,2}$ (Becton

Animals were maintained under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" by the Ministry of Education, Science and Culture, Japan.

Dickinson Co. Ltd., CA) or anti-mouse IgG (Bio Genex Lab., CA) by the immunoperoxidase method.

RESULTS

In the preliminary study, mice given 0 or 20 ppm azathioprine diet showed 100% survival at 12 weeks. However, the mice given 100 ppm azathioprine showed inhibition of body weight gain after the start of the experiment. One male mouse on 100 ppm diet died within 3 weeks and survivors were killed at 3 weeks. Table I summarizes body and organ weights and numbers of red (RBC) and white blood cells (WBC) at 3 weeks (100 ppm group) and 12 weeks (20 ppm group). In the 100 ppm group, thymus and spleen weights, and numbers of RBC and WBC were significantly decreased compared to the respective controls in both sexes. In the 20 ppm group, thymic weight in females and numbers of RBC and WBC in both sexes were significantly decreased compared to the respective controls.

In the chronic study, survivals at 60 weeks were 96% in group 1-a, 94% in group 1-b, 100% in group 1-c, 96% in group 2-a, 98% in group 2-b and 100% in group 2-c.

Increase of body weights among groups a, b and c in males was the same until about 70 weeks, when a plateau of about 35 g was reached. Final average body weights were 37.0 g in group 1-a, 35.7 g in group 1-b, 35.0 g in group 1-c. In females, average body weights increased steadily up to 60 weeks and thereafter gradually increased with small changes. Final average body weights were 35.4 g in group 2-a, 35.6 g in group 2-b and 34.6 g in group 2-c without any significant difference among them (Table II).

Azathioprine intake among groups a and b was fairly consistent. In groups 1-a and 2-a, it was around 2.5 mg/kg body weight/day in young animals, but it fell to about 1.6 mg after 50 weeks. In groups 1-b and 2-b, it was rather steady and ranged from 0.5 to 0.6 mg/kg body weight/day throughout life.

Table I. Changes in Organ Weights and Blood Cell Counts ofter Administration of Azathioprine for 3 Weeks (100 ppm) and 12 Weeks (20 ppm)

Sex	No. of mice	Azathioprine (ppm)	Body wt. (g)	Thymus (mg)	Spleen (mg)	Liver (g)	RBC ($\times 10^4$)	WBC ($\times 10^3$)
M	6	0	23.5±0.8	43.1±6.0	60.3±3.1	1.12±0.11	478±43	7.2±1.6
	6	100[a]	24.3±0.5	34.0±9.6*	56.4±2.7*	1.17±0.08	326±45**	2.4±0.3**
F	6	0	20.1±1.0	53.9±5.8	65.9±9.1	0.92±0.05	583±205	4.1±0.7
	6	100	19.3±1.0	28.7±2.3**	56.8±3.7**	0.90±0.07	235±83**	1.6±0.6**
M	5	0	24.6±1.3	31.4±4.2	59.4±6.7	0.97±0.06	630±17	6.3±1.5
	5	20[b]	25.2±1.3	29.8±1.7	55.4±7.4	1.12±0.04	568±11**	3.5±0.4*
F	5	0	22.0±1.0	44.2±6.0	78.1±10.2	0.88±0.06	682±32	6.9±1.0
	5	20	22.8±1.1	35.6±4.0*	72.4±4.3	0.86±0.03	636±15*	4.3±0.9**

a) Mice died at 4 to 5 weeks and others were killed 3 weeks after commencement of the diet containing 100 ppm of azathioprine.
b) Mice were killed 12 weeks after the commencement of the diet containing 20 ppm of azathioprine.
*, ** Significantly smaller than the control ($P<0.05$ and $P<0.01$, respectively, by Student's t test).

Table II. Experimental Design, Body, Thymus, Liver, Spleen and Uterine Weights in Mice Given Azathioprine for 100 Weeks

Exp. group	Sex	Azathioprine (ppm)	Effective no. of mice	Av. body wt. (g±SD)		Thymus (mg)	Liver (g)	Spleen (mg)	Uterus (g)
				Initial	Final				
1-a	M	20	48	21.1±1.3	37.0±4.8	22.8±9.8[a]	1.49±0.24	125±112	
1-b	M	5	47	22.5±1.6	35.7±3.1	22.8±9.8	1.45±0.16	107±5	
1-c	M	0	50	21.6±1.0	35.0±3.4	22.5±9.5	1.42±0.19	105±48	
2-a	F	20	48	17.6±1.0	35.4±6.2	26.1±10.1	1.25±0.23	158±112	1.39±2.21
2-b	F	5	49	18.2±1.0	35.6±6.3	27.1±7.5	1.28±0.20	200±164	1.06±1.16
2-c	F	0	50	17.4±1.3	34.6±5.0	25.9±8.4	1.35±0.40	180±104	0.72±0.76

a) Leukemic thymuses were excluded.

Table III. Number of Azathioprine-treated Mice Bearing Various Types of Tumors

Experimental group	1-a	1-b	1-c	2-a	2-b	2-c
Effective no. of mice	48	47	50	48	49	50
Tumor type						
Skin						
fibrosarcoma	0	0	0	1	0	0
Lung						
adenoma	1	3	2	0	0	0
carcinoma	1	0	0	0	0	0
Lymphoma						
systemic	1	3	0	6*	5	1
localized	2	1	1	0	2	1
Forestomach						
papilloma	2	0	2	3	1	3
Glandular stomach						
adenocarcinoma	1	0	0	0	0	0
Liver						
carcinoma	1	0	0	0	0	0
adenoma	1*	2	8	1	0	0
altered focus	1	0	0	0	0	0
hemangioma	0	0	0	0	0	1
Ovary						
granulosa cell T.				0	1	0
Mammary gland						
carcinoma				0	1	0
Harderian gland T.	0	1	0	0	0	0
Uterus						
hemangioendothelioma				7**	0	0
myosarcoma				0	1	0

*, ** Significantly different from the respective controls ($P<0.05$ and $P<0.01$, respectively) by chi-square test.

Occurrence of tumors is summarized in Table III. Among all experimental groups, lymphoma appeared at the highest frequency followed by papillomas in the forestomach, hepatic tumors and uterine tumors. Occurrence of hepatic adenomas in males was inversely related to the administered dose of azathioprine. Incidence in group 1-a (0.5%) was significantly smaller than that in group 1-c (16%), with $P<0.05$. In females, however, high frequencies of systemic lymphomas, papillomas in the forestomach and uterine tumors were noted. Among them, the occurrence of systemic lymphomas in group 2-a (12.5%) was significantly higher than that in group 2-c (0.5%), with $P<0.05$, and they appeared between 60 and 100 weeks. Similarly, occurrence of hemangioendotheliomas in group 2-a (14.6%) was significantly higher than that in group 2-c (0%), with $P<0.01$, and they were all found at the termination of the experiment at 100 weeks.

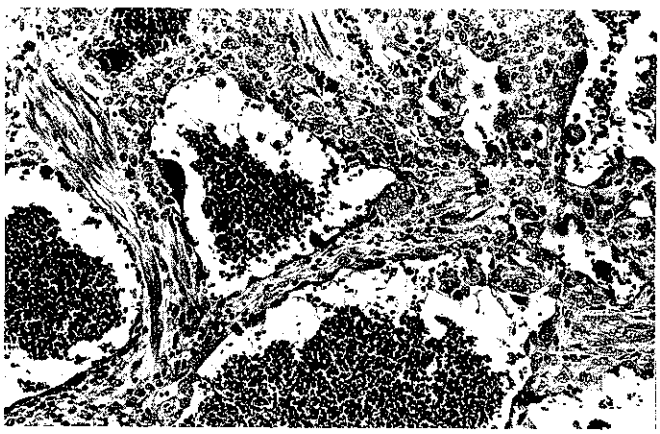

Fig. 1. A uterine hemangioendothelioma in a female mouse given 20 ppm azathioprine diet for 100 weeks. Massive blood congestion is seen in the vascular space and giant-nucleated cells appear among neoplastic endothelial cell proliferation. H-E stain, ×545.

Systemic lymphomas occurred exclusively in thymus, spleen and mesenteric lymph nodes, and occasional involvement was noted in liver, subcutaneous lymph nodes and Payer's patches in the small intestine. Microscopic examination revealed that they were composed of medium to large neoplastic lymphoid cells. Neoplastic foci in the thymus and lymph nodes were studied to determine their cellular character by immunohistochemical staining with anti-$Thy_{1,2}$ antibody. All of the neoplastic cells stained negatively with anti-$Thy_{1,2}$ antibody or anti-mouse immunoglobulin in contrast to the well stained intact lymphocytes.

Localized lymphomas were noted in the mesenteric lymph nodes and their occurrence did not show azathioprine dose-dependency.

Seven cases of hemangioendotheliomas in group 2-a (14.6%) each developed as a single tumor in a uterine horn associated with massive hemorrhage in distended blood vessels. Average uterine weight was azathioprine dose-dependent, but without statistical significance, since there was a high frequency of spontaneous hydrometras in this strain of aged mice. Uterine tumors protruded into the uterine cavity covered with endometrial cell layers. Microscopically, they were composed of cavernous vascular spaces filled with erythrocytes separated by irregular sheets and solid areas of polymorphic endothelial cells. There were also giant or multi-nucleated cells in the neoplastic area (Fig. 1). This type of tumor only appeared in group 2-a and no metastatic foci were detected in any organ. No preneoplastic lesions were noted in the uterine horn in other azathioprine-administered mice.

Lung adenomas in males were frequent and papillomas in the forestomach were observed with similar frequency in both sexes, but they did not show dose-dependence on azathioprine.

DISCUSSION

In the preliminary study, the concentration of 100 ppm was highly toxic and all mice died within the observation period due to loss of weight and immune deficiency. The dose of 20 ppm, however, was not found to be toxic for 12 weeks in the same experiment, although it had a suppressive effect on the hematopoietic tissues. In the long-term study, the dose of 20 ppm was successfully applied. By calculation from the food intake and changes in body weight, total azathioprine intake in male and female mice over their whole life would have been roughly 39.4 mg and 39.8 mg per mouse, respectively.

In the large-scale observation of spontaneous tumors in B6C3F1 mice, there was no report of hemangioendothelioma at the uterus, and the incidence of uterine sarcomas was only 0.07%.[12] Thus, occurrence of hemangioendotheliomas in the uterus reported here seemed to be related to the administration of azathioprine.

Occurrence of lymphomas was significantly higher in group 2-a than in group 2-c, even though the overall incidence remained low. Imamura *et al.* previously reported that concomitant administration of azathioprine with butylnitrosourea efficiently induced thymic lymphomas in C57BL mice.[13] Shinozuka *et al.* also extensively studied leukemogenesis by the immunosuppressant cyclosporin.[14–16] They also reported the enhanced occurrence of intestinal tumors in the case of combined treatment with methylnitrosourea and cyclosporin.[17] Clinical studies on the development of malignancies among organ transplant recipients indicated that either skin cancers or lymphomas appeared with the highest frequency among cancers in patients treated with either azathioprine or cyclosporin.[1, 2, 18]

On the other hand, the inhibitory effect of azathioprine on the spontaneous occurrence of hepatic tumors puzzled us. It may be worthwhile to study not only the tumorigenicity of this chemical but also the additive effect of this compound with other genotoxic agents.

In general, total tumor incidences in this experiment seemed to be low compared to those in previous or simultaneously conducted experiments in our laboratory using the same strain of mice. This may be due to the use of powdered diet, since the increases of average body weight in both sexes were less than those of mice that were fed with regular biscuit diet (A. Ito *et al.*, unpublished data).

It was concluded that azathioprine is tumorigenic to hematopoietic and uterine tissues. Conversely, the immunosuppresive state induced by azathioprine may also be suppressive for the spontaneous occurrence of liver tumors.

As to the human risk assessment of azathioprine, which was determined to be a carcinogen in human,[19] its administration to humans should be avoided or minimized. Assuming that mice and humans have a similar sensitivity to azathioprine, total oral administration should be less than 1.13 g/kg of body weight in males and 1.14 g/kg of body weight in females to minimize the risk of occurrence of neoplasms in humans.

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REFERENCES

1) Penn, I. Malignancies associated with immunosuppressive or cytotoxic therapy. *Surgery,* **83,** 492–502 (1978).
2) Penn, I. Cancers following cyclosporine therapy. *Transplantation,* **43,** 32–35 (1987).
3) Elion, G. B. Significance of azathioprine metabolites. *Proc. Roy. Soc. Med.,* **65,** 257–259 (1972).
4) Gilbett, E. R., Anderson, J. F., Cohen, F., Pollara, B. and Meuwissen, H. J. Adenosine deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet,* **II,** 1067–1069 (1972).
5) Cohen, S. M., Erturk, E., Skibba, J. L. and Bryan, T. Azathioprine induction of lymphomas and squamous cell carcinomas in rats. *Cancer Res.,* **43,** 2768–2772 (1983).
6) Demetris, A. J., Nalesnik, M. A., Kunz, H. W., Gill, T. J., III and Shinozuka, H. Sequential analysis of the development of lymphoproliferative disorders in rats receiving cyclosporin. *Transplantation,* **38,** 239–246 (1984).
7) Harvey, J. J., East, J. and Katz, F. E. Azathioprine induced lymphocytic neoplasms of NZB mice lack ecotropic murine leukemia virus. *Int. J. Cancer,* **23,** 217–223 (1979).
8) Mitrou, P. S., Fisher, M., Mitrou, G., Rottger, P. and Holtz, G. The oncogenic effect of immunosuppressive (cytotoxic) agents in (NZB×NZW) mice. I. Long term

treatment with azathioprine and ifosfamide. *Arzneim. Forsch.*, **29**, 483–488 (1979).
9) Ito, A., Naito, M., Naito, Y. and Watanabe, H. Tumorigenicity test of N-(5-nitro-2-furfurylidene)-1-aminohydantoin by dietary administration in BDF1 mice. *Hiroshima J. Med. Sci.*, **32**, 99–102 (1983).
10) Ito, A., Watanabe, H., Naito, M., Aoyama, H. Nakagawa, Y. and Fujimoto, N. Induction of thyroid tumors in (C57BL/6N×C3H/2N)F1 mice by oral administration of 9,3,4,5,6-tetrachloro-o-carboxyphenyl-6-hydroxy-2,4,5,7-tetraiodo-3-isoxanthone sodium (Food red 105, Rose Bengal B). *J. Natl. Cancer Inst.*, **77**, 277–281 (1986).
11) Naito, M., Ito, A., Watanabe, H., Kawashima, K. and Aoyama, H. Carcinogenicity of ortho-ethoxybenzamide in (C57BL/6N×C3H/2N)F1 mice. *J. Natl. Cancer Inst.*, **76**, 115–118(1985).
12) Ward, J. M., Goodman, D. G., Squire, R. A., Chu, K. C. and Linhart, M. S. Neoplastic and nonneoplastic lesions in aging (C57BL/6N×C3H/HeN)F1 (B6C3F1) mice. *J. Natl. Cancer Inst.*, **63**, 849–854 (1979).
13) Imamura, N., Nakano, M., Kawase, A., Kawamura, Y. and Yokoro, K. Synergistic action of N-nitrosobutylurea and azathioprine in induction of leukemia in C57BL mice. *Gann*, **64**, 493–498 (1973).
14) Hattori, A., Kunz, H. W., Gill, T. J., III, Pan, S. F. and Shinozuka, H. Diversity of the promoting action of cyclosporin on the induction of murine lymphoid tumors. *Carcinogenesis*, **9**, 1091–1094 (1988).
15) Hattori, A., Perera, M. I. R., Witkowski, L. A., Kunz, H. W., Gill, T. J., III and Shinozuka, H. Accelerated development of spontaneous thymic lymphomas in male AKR mice receiving cyclosporin. *Transplantation*, **41**, 784–787 (1986).
16) Shinozuka, H., Gill, T. J., III, Kunz, H. Z., Witkowski, L. A., Demetris, A. J. and Perera, M. I. Enhancement of the induction of murine thymic lymphomas by cyclosporin. *Transplantation*, **41**, 377–380 (1986).
17) Perera, M. I., Kunz, H. W., Gill, T. J., III and Shinozuka, H. Enhancement of induction of intestinal adenocarcinomas by cyclosporin in rats given a single dose of N-methyl-N-nitrosourea. *Transplantation*, **42**, 297–302 (1986).
18) Thomson, A. W. Immunobiology of cyclosporin — a review. *Aust. J. Exp. Biol. Med. Sci.*, **61**, 147–172 (1983).
19) IARC. Azathioprine. *In* "Some Antineoplastic and Immunosuppressive Agents," IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Man, Vol. 26. pp. 47–78 (1981). IARC, Lyon.