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Hepatocellular Tumorigenicity of Butylated Hydroxytoluene Administered Orally to B6C3F$_1$ Mice

Kouki INAI, Toshihiro KOBUKE, Shigeru NAMBU, Tsuyoshi TAKEMOTO, Eihaku KOU, Hajime NISHINA, Megumu FUJIHARA, Shuji YONEHARA, Shin-ichi SUEHIRO, Takafumi TSUYA, Kenji HORIUCHI and Shoji TOKUOKA

Department of Pathology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734

Butylated hydroxytoluene (BHT), a preservative widely found in food as a food additive, was orally administered at concentrations of 1% and 2% of the diet to B6C3F$_1$ mice for 104 consecutive weeks. Treated animals underwent a 16-week recovery period prior to pathological examination. In male mice administered BHT, the incidence of mice with either a hepatocellular adenoma or a focus of cellular alteration in the liver was increased in a clear dose-response relationship. The incidences of male mice with other tumors and the incidences of female mice with any tumor were not significantly increased as a consequence of BHT administration. The results of this study indicate BHT to be tumorigenic to the liver of the B6C3F$_1$ male mouse.

Key words: BHT — Hepatocellular tumor — B6C3F$_1$ mouse

Butylated hydroxytoluene (BHT) has been widely used as a food additive to prevent oxidative decomposition of dietary fats since 1954 in the United States and 1956 in Japan. In the United States, the average human consumption of BHT has been estimated to range from 0.1 to 0.8 mg/kg/day, and the acceptable daily intake has been determined to be 0.5 mg/kg/day by the Food and Agriculture Organization, World Health Organization.[1]

An early study revealed that while BHT had a low grade of toxicity, it induced enlargement of the liver in rats.[2] Subsequently in rats, BHT at levels of 0.3–0.6% in the diet for 108 wk was found to produce hepatocytomegaly, peliosis, hepatocellular degeneration and necrosis in a dose-related manner.[3] In mice, treatment with BHT at a level of 0.75% in the diet for 12 months produced bile duct hyperplasia.[4]

Some studies in which BHT was administered orally to mice or rats have not demonstrated significant tumorigenic effects.[3–7] However, recent studies by Olsen *et al.*,[8] Lindenschmidt *et al.*[9] and Moch[10] showed an enhanced development of hepatocellular tumor in mice or rats.

The results of various *in vitro* and *in vivo* tests for mutagenicity with BHT, including the Ames test, have been varied. In general, BHT has not been considered to be mutagenic despite a few reports to the contrary.[11–13] Recently it has been proposed that chronic injury to the liver following BHT administration induces hepatic tumors by non-genotoxic mechanisms.[14]

The present study was carried out to evaluate the chronic toxicity of BHT administered orally to B6C3F$_1$ mice. Particular attention was given to BHT's potential for hepatocellular tumorigenicity.

Materials and Methods

Subacute Toxicity Test Prior to the initiation of the chronic study, a subacute toxicity test was performed to establish the maximum tolerated dose (MTD) of BHT in B6C3F$_1$ mice. This MTD was then used in the chronic study. Seventy male and 70 female 4-week-old B6C3F$_1$ mice were purchased from Charles River Japan Inc. (Atsugi) and observed during a 4-week quarantine period. The 8-week-old mice were divided into five treatment groups, each consisting of 10 male and 10 female mice, and one control group which consisted of 20 male and 20 female mice. The treated groups were given *ad libitum* for 10 consecutive weeks a diet containing BHT at a level of 0.25%, 0.5%, 1%, 2% or 4% of the diet. The BHT utilized in this study had a purity of 96% and the mixture of BHT and basal diet (CRF-1, Charles River Japan Inc.) was made into pellets. By means of analysis of the

pellets, performed once at the National Institute of Hygienic Sciences, the actual level of BHT in the pellets was proved to be almost 50% of the initial content (unpublished data). The control group was fed the basal diet and all groups, treated and control, were given tap water *ad libitum*. Ten mice of the same sex were housed in a plastic cage; all cages were kept in the same air-conditioned room. At the end of the 10-week treatment period all surviving mice were anesthetized with ether, sacrificed and necropsied. All animal necropsies were conducted by pathologists experienced in rodent necropsy techniques.

All of the mice, except for one male in the 1% group and one male and three female mice in the control group that died accidentally, survived the entire treatment period. The average rates of body weight gain for the male and female mice given a diet containing 4% BHT were less than 90% of those of the control group. Histological examination of major visceral organs of mice that had been given the diet containing 4% BHT showed marked starvation atrophy of the spleen, heart and kidneys. No significant gross or histological changes were detected in major visceral organs of mice that had been given a diet containing 2% or lower concentrations of BHT, or in the mice of the control group. On the basis of these results, the MTD of BHT in the diet was estimated to be 2% for B6C3F$_1$ mice of both sexes.

Chronic Toxicity Test For the chronic toxicity test, B6C3F$_1$ mice (150 male and 150 female, 4 weeks old) were purchased from Charles River Japan Inc. After 4 weeks they were divided into two treated groups and one control group, each consisting of 50 males and 50 females. The doses of BHT administered were 2%(high dose) and 1% (low dose), levels chosen on the basis of the results of the subacute toxicity test. The mice were given BHT incorporated into the basal diet (CRF-1) in the same manner as in the subacute toxicity test and tap water *ad libitum* for 104 consecutive weeks. The control group was fed the basal diet and tap water. All of the mice were housed, five male or ten female mice to a plastic cage; all cages were housed in the same air-conditioned room.

The amount of the diet consumed per cage and the body weight of each mouse were measured once every other week for the first 12 weeks and then once every 4 weeks until the end of the 104-week treatment period. After the treatment period all of the surviving mice were given the basal diet for an additional 16 weeks.

Any mouse that was found dead or moribund during the experiment was necropsied. The mice which survived to the end of the 120-week experimental period (62 males and 81 females) were anesthetized with ether, sacrificed, and necropsied. At necropsy all major visceral organs and all tumors observed at this time were weighed and prepared for microscopic examination. The incidences of mice with various tumors and the survival times of the mice with tumors in the treated groups were compared with those of the control group, using the chi-square test or *t*-test.

Results

The average daily intakes of diet and BHT per mouse are shown in Table I. No significant differences in amount were noted between the daily intake of diet by male mice given BHT and that of the control mice. The intake of diet by female mice given BHT, however, was greater than that of the control mice. This was most likely caused by the spillage of food by female mice given BHT. The ratio of daily intake of BHT adjusted for body weight of mice at the high dose(2%) level compared to that of those at the low dose (1%) level was 2.1:1 for male mice and 2.4:1 for female mice.

The average body weights of both male and female mice given BHT showed a dose-related reduction in comparison with those of the control mice (Figs. 1 and 2).

The percent survivals at 4-week intervals are shown in Figs. 3 and 4. In female mice, six of the high dose level group and two of the low dose level group died accidentally in the early treatment period (before the 14th week) and were, therefore, excluded when determining the percent survivals for these groups. In male mice there was a dose-related change in survival throughout the treatment period. In female mice there was no difference in survival between mice given BHT and the control mice

Table I. Average Daily Intakes of Diet and BHT per Mouse

Sex	BHT dose (%)	Average diet intake (g/day)	Average BHT intake (mg/day) (g/kg body wt/day)
Male	0	5.9	0
	1	5.9	59 (1.64)
	2	5.8	116 (3.48)
Famale	0	4.3	0
	1	5.7	57 (1.75)
	2	5.9	118 (4.13)

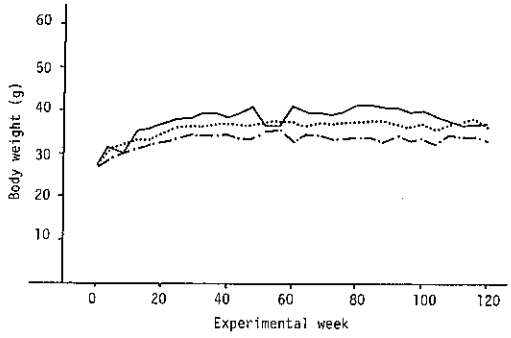


Fig. 1. Body weights of male mice of the 0% group (——), 1% group (------) and 2% group (– – –).

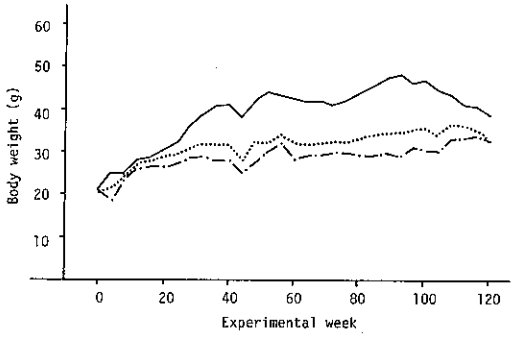


Fig. 2. Body weignts of female mice of the 0% group (——), 1% group (------) and 2% group (– – –).

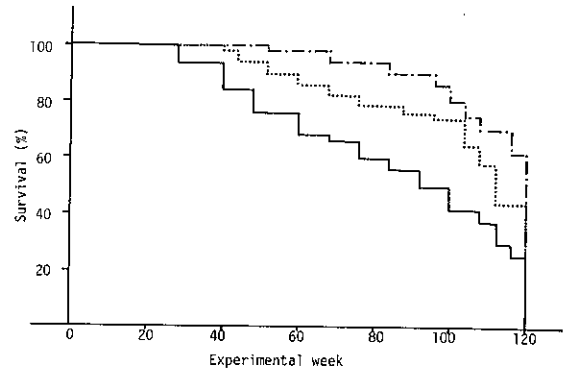


Fig. 3. Percent survival of male mice of the 0% group (——), 1% group (------) and 2% group (– – –).

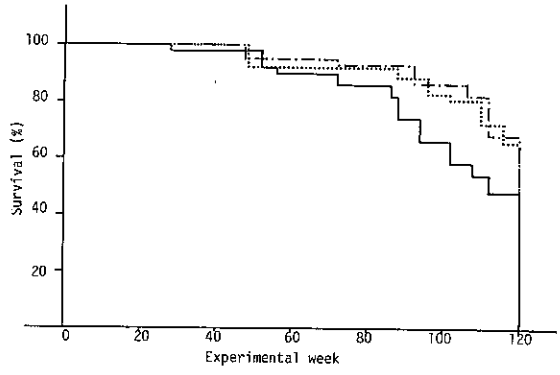


Fig. 4. Percent survival of female mice of the 0% group (——), 1% group (------) and 2% group (– – –).

until the 88th week of treatment. Thereafter, the survival of mice given BHT was higher than that of control mice. At the 104th week of treatment, the percent survivals for male mice were: high dose(2%), 74%, low dose (1%), 64% and control, 40%. For female mice, the survival rates were: high dose(2%), 89%, low dose(1%), 81% and control, 58%.

The mice that were regarded as "effective" for data analysis were those that survived beyond the 62nd week when, in the present study, the first mouse with a liver tumor died. Four male and 7 female mice that survived beyond the 62nd week were excluded from being considered "effective" because either tissue autolysis and/or cannibalism prevented adequate amounts of properly fixed tissue from being examined light microscopically. As shown in Table II, 89% of the male mice treated with BHT were "effective." The percentage of "effective" mice for the male control group was lower (64%) than that for the treated male mice given BHT. Likewise, that of female control mice (82%) was lower than that of female mice given BHT (91%).

The numbers of mice with tumors and the survival times of the mice with tumors are also shown in Table II. For female mice given BHT at the high dose level, the incidence of mice with tumors was significantly lower ($P<0.01$) and the survival time of mice with tumors was significantly longer ($P<0.01$) than that of control mice. For male mice the survival times of mice with tumors were not significantly different between mice given BHT and control mice.

Table II. Numbers of Effective Mice and Mice with Tumors

Sex	BHT dose (%)	No. of effective mice & average survival times (wk)[a]	No. and incidences (%) of mice with tumors & average survival times (wk)[a]
Male	0	32	27 (84)
		106±16	109±13
	1	42	36 (86)
		110±15	110±13
	2	47	38 (81)
		113±13	114±12
Female	0	41	35 (85)
		109±15	108±15
	1	44	33 (75)
		115±11	114±12
	2	40	22 (55)[b]
		118±5[c]	118±4[d]

a) Values are means±SD.
b) Significantly different from 0% group at $P<0.01$ (by chi-square test).
c) Significantly different from 0% group at $P<0.001$ (by *t*-test).
d) Significantly different from 0% group at $P<0.01$ (by *t*-test).

The incidences of mice with various histological types of tumors are shown in Table III. In female mice the tumor with the highest occurrence was lymphoma/leukemia. No tumor type, however, showed any statistically significant difference between mice given BHT and control mice. In male mice, the only statistically significant difference occurred when the number of mice treated with BHT having hepatocellular adenoma was compared to control mice with the same tumor ($P<0.01$).

Table IV provides a summary of the hepatocellular lesions identified in mice of both sexes. The hepatocellular lesions seen in this study were classified as described by Frith and Ward.[15] The lesions included hepatocellular carcinoma, hepatocellular adenoma and a focus of cellular alteration. The compression of the surrounding normal parenchymal tissue was a diagnostic feature used to separate hepatocellular adenoma from a focus of cellular alteration. Histological changes considered to be significant were limited to male mice. A clear dose-response relationship was apparent in the incidence of male mice with hepatocellular adenoma. This was particularly evident when the incidence of male mice with multiple hepatocellular adenomas was considered ($P<0.01$). The number of foci of cellular alteration per male mouse also revealed a dose-response relationship. The incidence of mice with a hepatocellular carcinoma showed no difference when treated male mice were compared to concurrent controls. In the sacrificed male mice at the end of experiment (the 120th week) the incidences of mice with hepatocellular tumor were: high dose(2%), 77% (23 out of 30 mice), low dose(1%), 62% (13/21) and control, 45% (5/11). For male mice that died before the end of experiment, the incidences were: high dose(2%), 47% (8/17); low dose(1%), 62% (13/21); control, 33% (7/21).

The histological findings seen in the liver of a representative male mouse given BHT at the high dose(2%) level are shown Figs. 5 and 6. A large hepatocellular adenoma is present, and in the parenchyma adjacent to this nodule, several foci of cellular alteration may be seen. These foci suggest structural abnormalities of the hepatic lobules and may serve as a background for tumor formation. A large hepatocellular carcinoma in the liver of a control male mouse is shown in Fig. 7. The adjacent parenchyma, however, was essentially normal.

Table III. Incidences of Mice with Various Histological Types of Tumors

Sites	Types of tumor	No. and incidences (%) of mice with tumors in					
		males at BHT doses			females at BHT doses		
		0%	1%	2%	0%	1%	2%
Liver	Hepatocellular adenoma	6 (19)	16 (38)	25 (53)[a]	5 (12)	7 (16)	2 (5)
	Hepatocellular carcinoma	7 (22)	11 (26)	8 (17)	2 (5)	1 (2)	
	Hemangioma	4 (13)	3 (7)	1 (2)			1 (3)
	Angiosarcoma			1 (2)			
Lung	Alveolar-bronchiolar adenoma	4 (13)	6 (14)	10 (21)	4 (10)	5 (11)	1 (3)
	Alveolar-bronchiolar carcinoma	1 (3)	3 (7)		1 (2)	2 (5)	2 (5)
Hematopoietic system	Lymphoma/leukemia	6 (19)	9 (21)	4 (9)	19 (46)	11 (25)	10 (25)
Spleen	Hemangioma	2 (6)	3 (7)	2 (4)	2 (5)	2 (5)	2 (5)
	Angiosarcoma	1 (3)	1 (2)			1 (2)	1 (3)
Integumentary system	Fibroma	1 (3)	1 (2)				
	Fibrosarcoma				1 (2)		
	Liposarcoma				1 (2)		
	Leiomyosarcoma		1 (2)			2 (5)	
	Malignant fibrous histiocytoma	2 (6)	1 (2)		1 (2)	1 (2)	
Uterus	Leiomyoma						1 (3)
	Leiomyosarcoma				3 (7)	1 (2)	3 (8)
	Stromal sarcoma				1 (2)	1 (2)	1 (3)
	Hemangioma					2 (5)	
Ovary	Granulosa cell tumor					1 (2)	
Breast	Fibroadenoma					1 (2)	
Pancreas	Adenocarcinoma		1 (2)				
	Mucoepidermoid carcinoma		1 (2)				
Esophagus	Papilloma					1 (2)	
Forestomach	Papilloma				1 (2)		
Intestine	Leiomyoma					1 (2)	
	Adenocarcinoma			1 (2)			
Brain	Meningioma			1 (2)			
Pituitary gl.	Adenoma				1 (2)		
Parathyroid gl.	Adenoma	1 (3)					
Heart	Hemangioma				1 (2)		
Eyelid	Sebaceous carcinoma				2 (5)		

a) Significantly different from 0% group at $P<0.01$ (by chi-square test).

Table IV. Incidences of Hepatocellular Lesions in Mice

Sex	BHT dose (%)	No. of effective mice	No. and incidences (%) of mice with hepatocellular tumors	No. of mice (%) with				No. of foci of cellular alteration (No./mouse)
				single adenoma	multiple adenomas	adenoma & carcinoma	carcinoma	
Male	0	32	12 (38)	3 (9)	2 (6)	1 (3)	6 (19)	1 (0.03)
	1	42	26 (62)	11 (26)	4 (10)	1 (2)	10 (24)	25 (0.60)[a]
	2	47	31 (66)	8 (17)	15 (32)[a]	2 (4)	6 (13)	42 (0.89)[a]
Famale	0	41	7 (17)	4 (10)	1 (2)	0	2 (5)	0
	1	44	8 (18)	7 (16)	0	0	1 (2)	1 (0.02)
	2	40	2 (5)	2 (5)	0	0	0	1 (0.03)

a) Significantly different from 0% group at $P<0.01$ (by chi-square test).

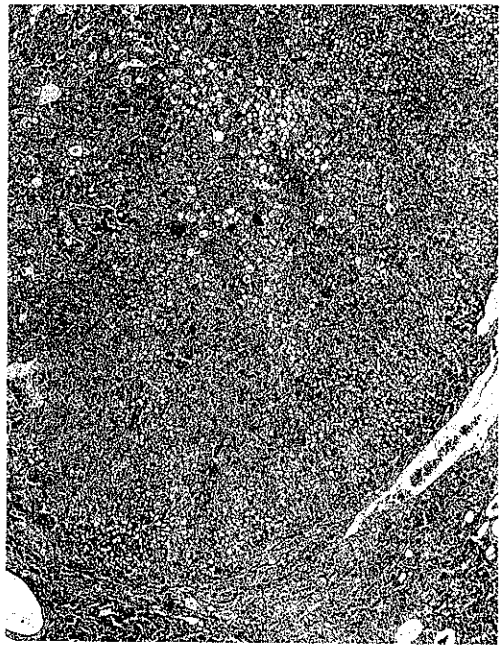


Fig. 5. Microscopic view of hepatocellular adenoma in the liver of a male mouse given BHT at the high dose level (2%). H-E stain. ×10. The tumor was associated with compression of the surrounding tissue.

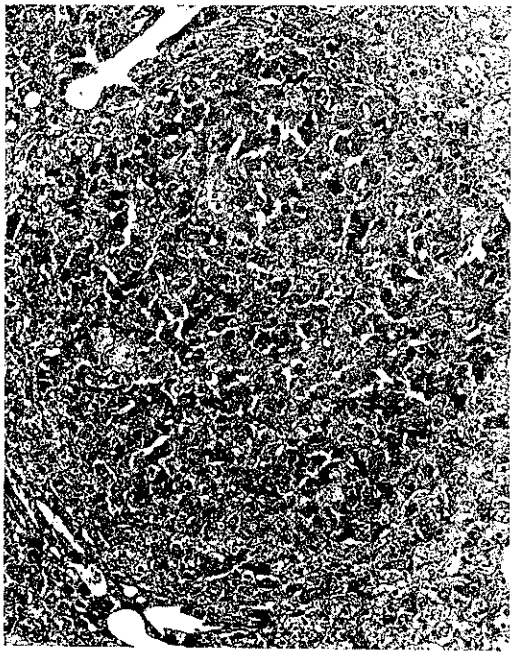
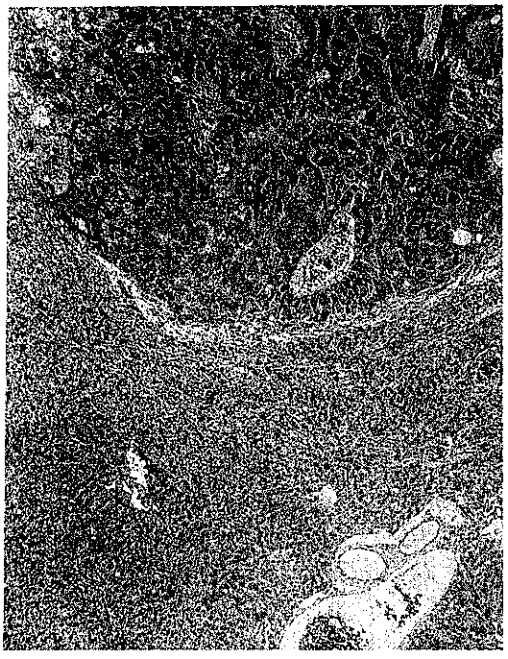


Fig. 6. Microscopic view of a focus of cellular alteration in the same liver as shown in Fig. 5. Note the absence of compression of the surrounding tissue. H-E stain. ×25.



The incidences of female mice with hepatocellular tumors were very low compared with those of male mice. Similarly, the livers of female mice had a lower incidence of foci of cellular alteration than did the livers of male mice.

The average liver weights of mice with and without hepatocellular tumors are shown in Table V. The average liver weight of all male mice (total mice) given BHT in each treatment group and their mean percentages of body weight were greater than those of control male mice. The average liver weight of male mice given BHT with no liver tumor was more than that of control mice. In female mice there was no significant difference in the liver weight of mice given BHT and that of

Fig. 7. Microscopic view of the liver of a male control mouse. A large hepatocellular carcinoma was noted and the surrounding liver parenchyma was essentially normal. H-E stain. ×13.

Table V. Average Final Body Weights and Liver Weights of Male Mice

BHT dose (%)	Group	No. of mice	Final body weight (g)[a]	Liver weight (g)[a]	Mean liver weight as percentage of body weight (%)[a]
0	Total mice	32	32.3±6.9	2.65±1.35	8.2±3.7
	Mice with HT[b]	11	32.8±6.5	3.41±1.22	10.7±3.7
	Mice with no tumor[c]	13	30.2±5.9	1.72±0.31	5.8±0.7
1	Total mice	42	33.9±5.6	3.65±1.68[e]	10.8±4.8
	Mice with HT	24	33.0±5.9	3.92±1.69	12.0±5.0
	Mice with no tumor	11	31.3±5.4	2.42±0.43[d]	7.9±1.4[d]
2	Total mice	47	31.1±4.7	3.35±1.54	10.8±4.8
	Mice with HT	30	32.1±4.1	3.77±1.59	11.9±5.2
	Mice with no tumor	11	28.8±5.1	2.40±0.73[e]	8.2±1.9[d]

a) Values are means±SD.
b) Mice with hepatocellular tumor, excluding mice with lymphoma/leukemia cell involvement in liver.
c) Mice with no liver tumor, including hepatocellular tumor, hemangioma, angiosarcoma and lymphoma/leukemia cell involvement.
d) Significantly different from 0% group at $P<0.001$ (by *t*-test).
e) Significantly different from 0% group at $P<0.01$ (by *t*-test).

control mice. In male mice given BHT, nuclear pleomorphism of hepatocytes was noted in non-tumorous areas, but hepatocellular necrosis, bile duct hyperplasia and peliosis were not seen.

As other pathological findings, male mice had a high incidence of amyloidosis that frequently affected the liver, spleen, kidney and/or adrenal gland. The incidences were 12.5% and 11.6% in mice given BHT at the high and low dose level, respectively, as compared with 27.3% in control mice. In female mice the incidence of amyloidosis was not remarkable in any group.

Discussion

The incidence of hepatocellular tumor in non-treated B6C3F$_1$ mice has been reported as 21.6% in males and 3.9% in females[16] or 31.1% in males and 7.9% in females.[17] In the previous experiments performed in our laboratory it was 32% in males and 2% in females.[18] In comparison with these reports, the incidence of hepatocellular tumor of control mice in the present study, 38% in males and 17% in females, was not so different, and therefore, the higher incidence of hepatocellular adenoma in male mice given BHT, associated with a dose-response relationship, strongly suggests that BHT is tumorigenic to the liver of B6C3F$_1$ male mice. The liver of female mice is considered not to be affected by BHT treatment in the present experiment. The reason for this sex difference is unknown, though in other reports[19, 20] on the tumorigenicity of chemical compounds only male mice of this strain showed enhancement of hepatic tumorigenesis, and it was suggested that male mice with a high incidence of spontaneous tumors responded more sensitively to carcinogens than female mice.

Up to the present, several studies on the tumorigenicity of BHT administered orally have been performed. Deichman *et al.*[5] reported that Wistar rats, given BHT orally for 24 months a high dose level of 1.0%, showed no evidence of tumorigenicity, although a reduction of body weight with an increase of mean percentage of liver and brain was observed. The studies reported by Clapp *et al.*[4] and that by the NIH[3] also showed no significant tumorigenicity of BHT. In Japan, Hirose *et al.*[6] found no tumorigenicity when BHT was administered to rats over a 24-month period at concentrations of 0.25% and 1%. Shirai *et al.*[7] found no tumorigenicity in B6C3F$_1$ mice administered BHT at concentrations of 5000, 1000 and 200 ppm.

The results of the present study are at variance with these reports. However, there are some points in which the experimental design of the present study differs from the previous ones. First, the high dose level, 2% chosen in the present study is higher than that in any of the previous reports; and furthermore the observation period, 120 weeks, is longer. These factors, therefore, might cause a masked weak tumorigenicity of BHT to become apparent. As supporting evidence for this, Olsen *et al.*[8] reported increased incidence of liver tumor in F_1 Wistar rats, which were delivered from parents administered BHT and in turn were administered BHT for 141–144 weeks; all these liver tumors were found when the F_1 rats had survived for more than 2 years. It seems therefore, that in Wistar rats with low spontaneous incidence of hepatocellular tumor, long-term exposure to BHT, including *in utero* exposure, was necessary to induce liver tumors.

Another study has claimed positive tumorigenicity of BHT. Lindenschmidt *et al.*[9] reported that C3H male mice, treated with 0, 0.05 and 0.5% BHT for 10 months showed the following incidences of liver tumors: 5% for the control, 58% for the low dose and 28% for the high dose group. It is, however, well known that the C3H mouse strain has a high and variable spontaneous incidence of liver tumor and therefore, it has been proposed that caution is necessary in the judgement of tumorigenicity of BHT.[14] Since the $B6C3F_1$ mouse strain used in the present study is F_1 of the C3H strain, we cannot necessarily conclude that BHT is tumorigenic simply on the basis of the occurrence of hepatocellular tumors in $B6C3F_1$ mice demonstrated in the present study.

In regard to the hepatocellular tumorigenicity of BHT, it remains uncertain whether BHT *per se* has an initiating activity or whether BHT plays a role as a promoter.[8] Most of the mutagenicity tests on BHT have demonstrated no genotoxic effect, and on the contrary,[11–13] it has been suggested by some investigators[21, 22] that a promotive effect of BHT is involved in hepatic tumorigenesis, as in the case of phenobarbital. Furthermore, many other reports[23–26] on chemical carcinogenesis in the urinary bladder, forestomach and lung have indicated a promotive activity of BHT. On the basis of the results in the present study, it cannot be determined whether the hepatocellular tumors were induced through either a promoting activity or an initiating activity of BHT, and further studies are necessary to ascertain this.

Powell *et al.*[14] suggested that the hepatocellular tumors were the consequence of chronic liver damage induced by BHT. The repeated cycle of necrosis and regeneration, observed in their study on short-term administration of high doses of BHT, might produce actively replicating cells, which are thought to be more vulnerable than resting cells to neoplastic transformation, either from spontaneous mutation or from environmental carcinogens. From this viewpoint, the lower body weight and the enlargement of liver in the male mice given BHT observed in the present study seem significant. The lower body weight of rats treated with BHT was reported by Olsen *et al.*,[8] who regarded it as a "toxic effect." The previous study[7] on $B6C3F_1$ mice given BHT, which showed no tumorigenic effect of BHT, indicated no significant reduction of body weight in the mice given BHT. An increase of liver weight induced by the administration of BHT was noted by some investigators.[2, 14, 22] As a cause, an activation of drug-metabolic enzymes in microsomes of the liver was suggested in the early study,[2] but it has been supposed recently that there is an acceleration of the normal age-related increase in ploidy, which means endonuclear reduplication, a form of hyperplasia.[14] From these facts, it seems that the lower body weight and the enlargement of liver are well correlated with hepatocellular tumorigenicity in the mice given BHT.

In the present study, a better survival of the mice given BHT was observed in spite of the lower body weight and the enlargement of the liver. The results were in accord with those reported by Harman,[27] Clapp *et al.*[28] and Olsen *et al.*[8] Yu *et al.*[29] suggested that the better survival might be related to the reduced body weight, but the cause of the better survival remains unclear. Moreover, most of the hepatocellular tumors in the present study were thought to have developed at a late stage of the experimental period, because the mice that survived longer showed a higher incidence of hepatocellular tumor. A decrease

with aging in the function of cytochrome 450, which is responsible for the metabolic breakdown of BHT in the liver, was regarded by Olsen *et al.*[8] as the reason why hepatocellular tumors were found in aged animals. This seems consistent with our results that most of the hepatocellular tumors were induced at a late stage of the experimental period. If it is assumed that tumors induced by BHT administration did not have time to develop, this would account for the lack of a statistically significant difference in the incidence of hepatocellular carcinoma between the mice given BHT and the control mice.

A suppressive effect of BHT in the process of experimental chemical carcinogenesis has been reported. The induction of mammary tumor in SD rats administered 7,12-dimethylbenz[*a*]anthracene,[30, 31] that of liver tumor in rats administered *N*-2-fluorenylacetamide[32] and that of intestinal tumor in rats administered azoxymethane[33] were reported to be suppressed by BHT. In the present study the number of female mice with tumors in the high dose(2%) group was significantly lower than that in the control group. The largest difference was in the incidences of lymphoid leukemia, although this was not statistically significant. The above result might suggest a tumor-suppressive effect of BHT. However in comparison with the results of previous reports, giving 16.8%[16] or 27.2%[17] as the incidence of lymphoid leukemia in non-treated female B6C3F$_1$ mice, the incidence in female control mice in the present study, 46%, was higher, while the incidence in female mice given BHT, 25%, was in accord with that mentioned in the previous reports. It is considered, therefore, that the present results do not indicate a suppressive effect of BHT on carcinogenesis.

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