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6-3. 1,1-ビス(tert-ブチルジオキシ)-3,3,5-トリメチルシクロヘサンのがん原性試験

CARCINOGENICITY STUDY OF 1,1-BIS(*tert*-BUTYLPEROXY)-3,3,5-TRIMETHYLCYCLOHEXANE IN B6C3F$_1$ MICE

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Abstract—1,1-Bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane (BBTC) is widely used in the manufacture of rubber. The present carcinogenicity study in B6C3F$_1$ mice was carried out in order to assess its potential to induce tumours. BBTC was administered at dietary levels of 0 (control), 0.25 and 0.5% for 78 wk; these dose levels were selected on the basis of a subchronic toxicity study, in which body weights were depressed to less than 90% of the control group values and swelling of hepatocytes was histologically evident in animals fed 1% BBTC or more in the diet. Neoplasms were found in all groups, including the control group, but there were no significant differences between groups of either sex in mortality, tumour incidences or tumour distribution. All tumours were considered to be spontaneous because of the similarity to background data for B6C3F$_1$ mice. This study thus provides no evidence of carcinogenicity of BBTC in B6C3F$_1$ mice.

INTRODUCTION

1,1-Bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane (BBTC) is widely used as a source of free radicals in the hardening of unsaturated polyester resins and the polymerization of styrene, finding particular application in the rubber industry. Its chemical structure is illustrated in Fig. 1. BBTC is not mutagenic in *Salmonella typhimurium* (E. Machigaki, personal communication, 1987). Although lauroyl peroxide (another source of free radicals used as an initiator in the polymerization of vinyl chloride in rubber manufacture) has also been shown not to be mutagenic in *S. typhimurium* (Yamaguchi and Yamashita, 1980), this compound has been suspected from bioassay data to have carcinogenic potential (Kotin and Falk, 1963). In addition, other free radical sources in the plastics and rubber industries such as *tert*-butylperoxy benzoate (Kotin and Falk, 1963) and benzoyl peroxide (Slaga *et al.*, 1981) have been shown to exert skin tumour-promoting activities or to be suspected carcinogens in preliminary animal studies.

Because BBTC has not been sufficiently examined for its possible toxicity and carcinogenicity despite its wide industrial use, the present investigation was carried out to assess any carcinogenic potential of the compound. This study was performed as a part of the risk re-evaluation of existing chemicals in Japan.

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Abbreviation: BBTC = 1,1-bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane.

MATERIALS AND METHODS

Animals

Male and female B6C3F$_1$ mice, purchased at the age of 5 wk from Charles River Japan Inc. (Kanagawa, Japan), were maintained on basal diet (MF; Oriental Yeast Ind. Co., Tokyo, Japan) and tap water until they were 6 wk old, when the studies started.

Chemical

BBTC (CAS No. 6731-86-8), purchased from Nippon Yushi Co. (Tokyo, Japan), was in a liquid form and was over 90% pure. It was administered orally to animals in the diet as detailed below. The diet supplemented with BBTC was kept at 4°C.

Housing conditions

Mice were housed 10 to a plastic cage, with softwood chips as bedding. The room was maintained at a temperature of 23 ± 2°C and a humidity of 55 ± 5%, with a 12-hr light/dark cycle.

Experimental design

A preliminary subchronic toxicity study was carried out prior to the carcinogenicity study.

Subchronic toxicity study. BBTC was added to MF powdered basal diet and fed *ad lib.* to groups of 10 male and 10 female mice at dietary concentrations of 0.5, 1.0, 2.0 or 4.0% for 13 wk. Control animals received the basal diet without BBTC. Throughout the experiment, mice were given tap water *ad lib.* Animals were observed daily for clinical signs and

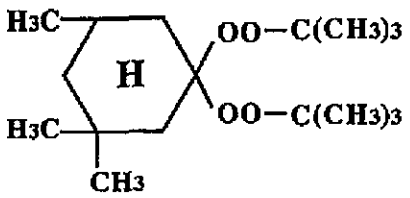

Fig. 1. Chemical structure of 1,1-bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane (BBTC).

deaths were recorded. At the end of the experiment, all surviving mice were killed, and major organs/tissues were taken for gross and microscopic examination. The results were used to determine appropriate dose levels for the subsequent carcinogenicity study.

Carcinogenicity study. Mice were divided randomly into three groups, each consisting of 50 males and 50 females. BBTC was added to the powdered basal diet at 0 (control), 0.25 or 0.5%. These dose levels were selected according to the results of the subchronic toxicity study. Animals were given their respective diet *ad lib.* for 78 wk, and the amounts of food consumed were measured in order to calculate the actual intakes of BBTC. Throughout the experiment, mice had free access to tap water. All mice were observed daily for clinical signs and deaths were recorded. Body weights were measured once a week for the first 13 wk of the study and then once every 4 wk. After 78 wk, the administration of BBTC was stopped and mice were then maintained on the powdered basal diet until wk 83 when all surviving animals were killed. All mice found dead, killed when moribund or killed at the end of the study were completely autopsied, and their organs were fixed routinely in 10% buffered formalin, sectioned and stained with haematoxylin and eosin.

Statistical analysis. Data were analysed for statistical significance by Fisher's exact probability test and the chi-square test.

RESULTS

Subchronic toxicity study

Two males and two females given 4.0% BBTC died during the study, all other mice survived until wk 13. Throughout the experiment, body weight gain and food consumption in the BBTC-treated groups were lower than those of the controls. For both sexes, the only dose of BBTC at which final body weights were in excess of 90% of the control values was 0.5%. Haematological examinations showed a tendency of anaemia in groups of both sexes receiving 1.0% BBTC or more. Relative liver weights were significantly increased in BBTC-treated mice in a dose-dependent manner. In contrast, absolute and relative spleen weights were decreased in a dose-dependent manner. Histopathological examinations revealed swelling of hepatocytes in male and female mice fed 1.0% BBTC or more, and atrophy of the red and white pulp in the spleen as well as a decrease of haematopoietic cells in the bone marrow were observed in males given 2.0 or 4.0% BBTC and in females fed 4.0% BBTC. From these results it was concluded that, with particular consideration given to growth retardation and histopathological findings, the maximum long-term dose of dietary BBTC that can be tolerated would be 0.5% for mice of both sexes. Therefore, 0.25 and 0.5% were selected as

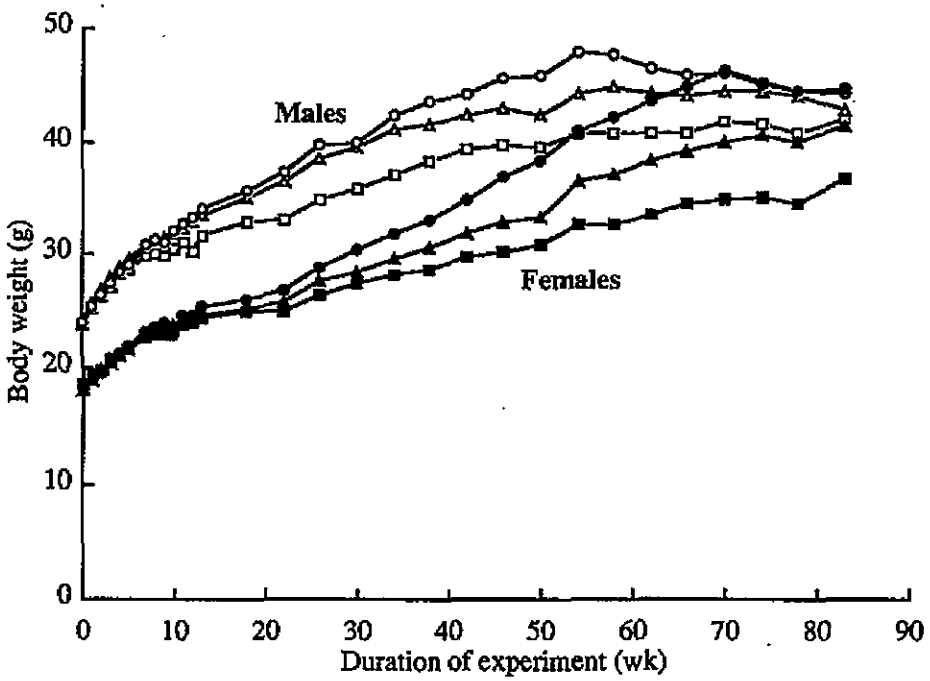

Fig. 2. Growth curves of B6C3F$_1$ mice given 1,1-bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane in the diet for 78 wk at 0 (males, ○; females, ●), 0.25 (males, △; females, ▲) or 0.5% (males, □; females, ■). Surviving mice were observed for a further 10 wk and killed at 83 wk.

Table 1. Total tumour incidences, food consumption, 1,1-bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane (BBTC) intake, final survival rate and mean survival time of B6C3F$_1$ mice given BBTC in the diet for 78 wk

BBTC dose (%)	No. of mice			Food consumption (g/animal/day)	Mean total BBTC intake (g/kg body weight/78 wk)	Final survival rate (%)	Mean survival time and range (wk)
	Initial	Effective	With tumour				
Males							
0	50	49	29	5.6	0	98.0	83.0 (82–83)
0.25	50	48	30	5.1	187	95.8	80.8 (23–83)
0.5	50	50	30	4.7	373	94.0	82.4 (61–83)
Females							
0	50	50	19	6.3	0	94.0	80.6 (15–83)
0.25	50	49	18	6.1	280	94.8	81.9 (47–83)
0.5	50	50	21	5.9	576	96.0	82.7 (73–83)

appropriate dose levels for the subsequent carcinogenicity study.

Carcinogenicity study

Growth and mortality. The growth curves (Fig. 2) showed a dose-dependent inhibitory effect of BBTC on the growth of mice of both sexes in the 0.25 and 0.5% groups. The survival rates and mean survival times (Table 1), however, indicated no significant differences between groups of either sex.

Tumour incidence and BBTC intake. Overall tumour incidences and total intakes of BBTC are summarized in Table 1. There were no significant differences in total tumour incidences between groups of either sex. Total intakes of BBTC, estimated from the food consumption data, were dose related.

Distribution and histopathology. The sites, histological types and incidences of tumours in each group are summarized in Table 2. Tumours were found in various organs from mice of both sexes in each group, including the control group. However, all the tumours were considered to be spontaneous because their incidences were essentially similar to those of spontaneous neoplastic lesions reported previously in

Table 2. Sites and types of tumours in B6C3F$_1$ mice given 1,1-bis(*tert*-butylperoxy)-3,3,5-trimethylcylohexane (BBTC) in the diet for 78 wk

Site and type of tumour	No. of mice with tumours					
	Males			Females		
BBTC dose (%) ...	0	0.25	0.5	0	0.25	0.5
Effective no. of mice	48	47	50	47	47	50
Lung						
Alveolar/bronchiolar adenoma	1	2	1	1	1	1
Alveolar/bronchiolar carcinoma	5	4	0*	0	1	0
Spleen						
Haemangioma	1	0	0	0	0	0
Haemangiosarcoma	1	0	0	1	0	0
Haematopoietic system						
Lymphoma	6	5	8	13	12	11
Histiocytic sarcoma	1	0	0	0	2	2
Small intestine						
Adenoma	0	1	0	0	0	0
Adenocarcinoma	0	1	0	0	0	0
Liver						
Hepatocellular adenoma	15	20	20	0	0	0
Hepatocellular carcinoma	8	7	7	0	0	0
Haemangioma	0	1	0	0	0	0
Haemangiosarcoma	1	1	0	0	0	0
Pancreas						
Acinar cell adenoma	0	0	0	0	0	1
Islet cell adenoma	1	1	0	0	0	0
Kidney						
Renal cell carcinoma	1	0	0	0	0	0
Adrenal gland						
Phaeochromocytoma	0	0	2	0	0	1
Cortical adenoma	0	0	0	0	1	1
Thyroid gland						
Follicular cell adenoma	0	0	0	1	0	0
Pituitary gland						
Adenoma (pars distalis)	0	0	0	1	0	0
Uterus						
Endometrial stromal polyp	—	—	—	1	0	0
Endometrial stromal sarcoma	—	—	—	1	1	2
Harderian gland						
Adenoma	5	3	0*	2	2	4
Adenocarcinoma	1	0	1	0	0	1
Skin/subcutis						
Schwannoma, malignant	0	1	0	0	0	0
Mastocytoma	0	0	0	0	0	1

*Significantly different from control group ($P < 0.05$).

Table 3. Incidences of total tumours and malignant tumours in B6C3F$_1$ mice given 1,1-bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane (BBTC) in the diet for 78 wk

Parameter	BBTC dose (%)	Males			Females		
		0	0.25	0.5	0	0.25	0.5
Effective no. of mice		49	48	50	50	49	50
No. of mice with tumours		29	30	30	19	18	21
Tumours/animal		0.94	0.94	0.78	0.42	0.41	0.48
No. of mice with malignant tumours		22	16	15	14	15	15
Malignant tumours/animal		0.49	0.39	0.32	0.30	0.33	0.30

B6C3F$_1$ mice (Tamano *et al.*, 1988; Ward *et al.*, 1979). BBTC treatment did not increase the incidences of any benign or malignant tumours (Table 3). Although the incidences of lymphomas and those of lung and Harderian gland tumours in both sexes, and liver tumours in males were relatively high in the control group compared with background data, there were no significant differences. Interestingly, the incidences of lung carcinomas and Harderian gland adenomas in male mice were decreased in a dose-dependent manner with statistical significance in the high-dose group.

Non-neoplastic lesions. Although non-neoplastic lesions were observed frequently in all groups, including the controls, no significant differences were found between groups. Swelling of centrilobular hepatocytes, as observed in the subchronic toxicity study, was evident only in male mice fed 0.5% BBTC.

DISCUSSION

Tumours of the liver, haematopoietic organs, lung and Harderian gland are known to develop spontaneously in mice of the B6C3F$_1$ strain (Tamano *et al.*, 1988; Ward *et al.*, 1979). In the present study, BBTC administration neither increased the incidences of such spontaneous tumours nor induced any unusual neoplasms. Slight but significant decreases in the incidences of lung carcinomas and Harderian gland adenomas were associated with BBTC treatment. With regard to lung carcinomas, similar results have previously been reported for cyclohexane (Lijinsky and Kovatch, 1986). The present results therefore suggest that BBTC may inhibit directly the development of some spontaneous tumours; however, the dose-dependent decreases in food consumption and body weight gain in the BBTC-treated groups may have acted as factors that suppress tumour development. Based on the fact that the incidences of both lung and Harderian gland tumours in the control group were elevated compared with earlier background data (Tamano *et al.*, 1988; Ward *et al.*, 1979), together with the finding that the total tumour incidences were similar to those found in previous studies (Tamano *et al.*, 1988; Ward *et al.*, 1979), the inhibitory effects were likely to be of little significance, if any.

Peroxides are widely used as a source of free radicals in various industries. Recently, free radicals have been suggested to play important biological roles, especially in carcinogenic processes. In fact, some peroxides such as *tert*-butylperoxy benzoate and benzoyl peroxide, which are functionally similar to BBTC, are known to be mutagenic (Mortelmans *et al.*, 1986; Saladino *et al.*, 1985) and carcinogenic (Kotin and Falk, 1963) or co-carcinogenic (Slaga *et al.*, 1981). The hepatotoxicity and haematotoxicity of BBTC were noted in the present subchronic toxicity study, but no nephrotoxicity was observed, despite the finding that cyclohexane and tetramethylcyclohexanes, which have structural resemblances to BBTC, are nephrotoxic in rats (Bernard *et al.*, 1989; Johannsen and Levinskas, 1987). The observed hepatotoxicity could have been caused by the induction of cytochrome *P*-450 enzyme activity, since it has been shown that the structurally similar hexachlorocyclohexane induces this activity in the liver (Popp and Cattley, 1991). Persistent induction of the cytochrome *P*-450 enzyme may give rise to subsequent hepatocarcinogenesis. The haematotoxicity might have been caused primarily by the damage of the haematopoietic organs, although nutritional impairment could, to some extent, contribute to its occurrence. Despite the cytotoxicity in the liver and haematopoietic organs, there were no significant increases caused by BBTC in the incidences of neoplasms in these organs.

It was therefore concluded that BBTC exerts no carcinogenic activity in B6C3F$_1$ mice. However, while cyclohexane has been suspected as a mutagen from the results of DNA–cell binding assays (Kubinski *et al.*, 1981) and is also known to be a skin tumour promoter in mice, it is not a complete carcinogen (Gupta and Mehrottra, 1990). Thus, although BBTC has been shown not to be mutagenic in the Ames test, the possibility that it can act as a tumour promoter requires further elucidation.

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6-4. 1,1-ビス(tert-ブチルジオキシ)-3,3,5-トリメチルシクロヘサン のその他の毒性情報

他の毒性情報	[三井ら：衛生試験所報告,110, 42-47,1992 より引用] 反復投与毒性試験 B6C3F$_1$ マウス　(0.5, 1.0, 2.0, 4.0%(混餌投与))　13 週間　純度 90%以上 {♂：800, 1500, 3200, 6000 mg/kg/day 相当、 ♀：1000, 1700, 3100, 6500 mg/kg/day 相当} NOEL：<0.5%(800 mg/kg/day) 死亡(4.0：♂8/10・♀8/10) 一般状態（削痩：2.0 以上♂♀） 体重↓(1.0 以上♂♀) 摂餌量↓：0.5 以上♂♀ 血液学的検査(Hgb↓・MCV↓：2.0♂♀、Hct↓・WBC↓：2.0♂) 相対重量(肝↑：0.5 以上♂♀) 病理組織学的所見(肝－小葉中心性細胞肥大：1.0 以上♂♀、 骨髄－造血細胞減少／うっ血：2.0 以上♂♀、 脾－白脾髄／赤脾髄萎縮：2.0 以上♂・4.0♀)