6. S9 mix

エームステスト用凍結S9 mix(キッコーマン㈱)を購入し，製造後6ヶ月以内に使用した．S9は，誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したprague-Dawley系雄ラットの肝臓から調製されたものである．

7. 試験方法

試験は，プレインキュベーション法で行った．

試験管に使用溶媒0.05 mL，被験物質供試液0.05 mLあるいは陽性対照物質溶液0.1 mLを入れ，次いで直接法では0.1 Mリン酸ナトリウム緩衝液(pH 7.4)を0.5 mL，代謝活性化法ではS9 mixを0.5 mL加え，続いて試験菌液0.1 mLを分注し，37 ℃で20分間振盪培養した．培養終了後，45 ℃に保温したトップアガー2 mLを加えた混合液をプレート上に重層した．37 ℃で48時間培養後，復帰変異コロニーを計数し，同時に指標菌株の生育阻害の有無を実体顕微鏡を用いて観察した．プレートは，用量設定試験では各用量とも1枚，本試験では3枚を使用した．本試験は，同一用量を用いて2回行った．

8. 結果の判定

被験物質処理プレートにおける復帰変異コロニー数(平均値)が溶媒対照値の2倍以上を示し，用量依存性および結果の再現性が認められる場合を陽性とした．

但し，明確な用量依存性が認められない場合においても，陽性値を示す試験結果に再現性が認められれば陽性と判定することとした．

結果および考察

20～5000 μg/plateの範囲で行った用量設定試験においては，代謝活性化の有無にかかわらず，いずれの菌株においても菌の生育阻害は認められなかった．したがって，本試験における被験物質の用量は，最高用量を5000 μg/plateとし，以下公比2で，2500，1250，625および313 μg/plateとした．

試験を2回行った結果(Tables 1～4)，直接法および代謝活性化法のいずれの場合も，供試した全ての菌株において復帰変異コロニー数は，溶媒対照値の2倍を越えることはなく，また，菌の生育阻害も認められなかった．

なお，代謝活性化の有無にかかわらず，1250 μg/plate以上の用量で培養終了時，プレート上に被験物質と思われる油滴様物が認められた．

以上の成績から，本実験条件下では，1,1-ビス(tert-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサンの遺伝子突然変異誘発性は陰性と判定した．

なお，1,1-ビス(tert-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサンおよびその類縁化合物の変異原性に関する報告は見当たらない．

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Table 1 Results of reverse mutation test of 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane on bacteria (1st trial) [direct method: -S9 mix]

Test substance dose (μg/plate)	Number of revertant colonies per plate [Mean ± S.D.]														
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537		
0	127	129	119	15	14	13	18	17	23	29	21	18	10	9	8
	[125 ± 5]			[14 ± 1]			[19 ± 3]			[23 ± 6]			[9 ± 1]		
313	120	108	126	17	11	12	14	24	18	26	21	19	11	8	9
	[118 ± 9]			[13 ± 3]			[19 ± 5]			[22 ± 4]			[9 ± 2]		
625	136	130	137	13	14	15	16	19	27	18	24	21	12	15	15
	[134 ± 4]			[14 ± 1]			[21 ± 6]			[21 ± 3]			[14 ± 2]		
1250[a]	122	118	120	10	9	5	23	17	17	26	19	40	8	11	8
	[120 ± 2]			[8 ± 3]			[19 ± 3]			[28 ± 11]			[9 ± 2]		
2500[a]	130	138	132	13	9	11	21	18	17	21	29	30	11	9	15
	[133 ± 4]			[11 ± 2]			[19 ± 2]			[27 ± 5]			[12 ± 3]		
5000[a]	128	118	134	6	7	7	17	16	16	30	26	25	14	17	9
	[127 ± 8]			[7 ± 1]			[16 ± 1]			[27 ± 3]			[13 ± 4]		
Positive control	847	782	760[a]	390	432	400[b]	714	697	746[c]	462	486	446[d]	516	520	703[e]
	[796 ± 45]			[407 ± 22]			[719 ± 25]			[465 ± 20]			[590 ± 99]		

#: Precipitate was observed on the surface of agar plates.
a) AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, 0.01 μg/plate
b) NaN_3: Sodium azide, 0.5 μg/plate
c) AF-2, 0.04 μg/plate
d) AF-2, 0.1 μg/plate
e) 9-AA: 9-Aminoacridine, 80 μg/plate

Table 2 Results of reverse mutation test of 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane on bacteria (1st trial) [activation method: +S9 mix]

Test substance dose (μg/plate)	Number of revertant colonies per plate [Mean ± S.D.]														
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537		
0	118	127	114	11	15	14	24	20	17	37	35	27	11	10	15
	[120 ± 7]			[13 ± 2]			[20 ± 4]			[33 ± 5]			[12 ± 3]		
313	117	131	130	13	13	14	20	27	18	46	46	40	18	11	12
	[126 ± 8]			[13 ± 1]			[22 ± 5]			[44 ± 3]			[14 ± 4]		
625	117	105	132	13	14	17	21	21	22	35	18	34	13	16	12
	[118 ± 14]			[15 ± 2]			[21 ± 1]			[29 ± 10]			[14 ± 4]		
1250[a]	122	121	121	16	11	12	21	17	26	31	32	31	7	12	15
	[121 ± 1]			[13 ± 3]			[21 ± 5]			[31 ± 1]			[11 ± 4]		
2500[a]	123	122	105	7	7	8	23	22	30	32	29	27	14	14	7
	[117 ± 10]			[7 ± 1]			[25 ± 4]			[29 ± 3]			[12 ± 4]		
5000[a]	134	139	106	6	6	10	30	22	28	38	25	20	15	10	9
	[126 ± 18]			[7 ± 2]			[27 ± 4]			[28 ± 9]			[11 ± 3]		
Positive control	689	718	671[a]	150	159	156[b]	820	829	864[c]	411	357	402[a]	111	114	125[b]
	[693 ± 24]			[155 ± 5]			[838 ± 23]			[390 ± 29]			[117 ± 7]		

#: Precipitate was observed on the surface of agar plates.
a) AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, 0.01 μg/plate
b) 2-AA, 2 μg/plate
c) 2-AA, 10 μg/plate

Table 3 Results of reverse mutation test of 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane on bacteria (2nd trial) [direct method: −S9 mix]

Test substance dose (μg/plate)	Number of revertant colonies per plate [Mean ± S.D.]														
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537		
0	98	100	122	12	10	14	15	18	21	30	28	27	8	8	10
	[107 ± 13]			[12 ± 2]			[18 ± 3]			[28 ± 2]			[9 ± 1]		
313	105	112	131	9	12	8	20	18	15	25	32	33	14	12	12
	[116 ± 13]			[10 ± 2]			[18 ± 3]			[30 ± 4]			[13 ± 1]		
625	110	90	108	9	13	10	22	17	16	30	31	31	14	11	13
	[103 ± 11]			[11 ± 2]			[18 ± 3]			[31 ± 1]			[13 ± 2]		
1250#	107	108	129	8	5	5	12	14	16	14	27	22	6	11	7
	[115 ± 12]			[6 ± 2]			[14 ± 2]			[21 ± 7]			[8 ± 3]		
2500#	121	83	109	6	6	9	16	25	14	17	21	24	12	10	8
	[104 ± 19]			[7 ± 2]			[18 ± 6]			[21 ± 4]			[10 ± 2]		
5000#	119	91	112	9	9	14	15	15	14	32	26	32	7	14	10
	[108 ± 13]			[11 ± 3]			[15 ± 1]			[30 ± 3]			[10 ± 4]		
Positive control	795	783	829[a]	386	358	336[b]	716	806	691[c]	472	488	402[d]	428	512	557[e]
	[802 ± 24]			[360 ± 25]			[738 ± 60]			[454 ± 46]			[499 ± 65]		

#: Precipitate was observed on the surface of agar plates.
a) AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, 0.01 μg/plate
b) NaN_3: Sodium azide, 0.5 μg/plate
c) AF-2, 0.04 μg/plate
d) AF-2, 0.1 μg/plate
e) 9-AA: 9-Aminoacridine, 80 μg/plate

Table 4 Results of reverse mutation test of 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane on bacteria (2nd trial) [activation method: +S9 mix]

Test substance dose (μg/plate)	Number of revertant colonies per plate [Mean ± S.D.]														
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537		
0	116	105	102	9	7	10	25	22	21	41	25	35	16	11	10
	[108 ± 7]			[9 ± 2]			[23 ± 2]			[31 ± 8]			[12 ± 3]		
313	127	105	120	7	13	8	21	30	10	27	33	44	16	13	19
	[117 ± 11]			[9 ± 3]			[20 ± 10]			[35 ± 9]			[16 ± 3]		
625	118	118	115	10	8	12	31	24	21	31	32	29	10	15	13
	[117 ± 2]			[10 ± 2]			[25 ± 5]			[31 ± 2]			[13 ± 3]		
1250#	128	128	104	13	11	7	12	28	26	40	28	36	13	15	14
	[120 ± 14]			[10 ± 3]			[22 ± 9]			[35 ± 6]			[14 ± 1]		
2500#	120	100	118	9	15	6	24	19	25	23	30	45	14	18	15
	[113 ± 11]			[10 ± 5]			[23 ± 3]			[33 ± 11]			[16 ± 2]		
5000#	93	131	101	13	11	6	20	24	17	33	23	23	6	13	10
	[108 ± 20]			[10 ± 4]			[20 ± 4]			[26 ± 6]			[10 ± 4]		
Positive control	699	668	623[a]	214	193	184[b]	685	723	803[c]	339	382	362[a]	173	121	142[b]
	[663 ± 38]			[197 ± 15]			[737 ± 60]			[361 ± 22]			[145 ± 26]		

#: Precipitate was observed on the surface of agar plates.
a) 2-AA: 2-Aminoanthracene, 1 μg/plate
b) 2-AA, 2 μg/plate
c) 2-AA, 10 μg/plate

1,1-ビス(*tert*-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサンのチャイニーズ・ハムスター培養細胞を用いる染色体異常試験

In Vitro Chromosomal Aberration Test of 1,1-Bis(*tert*-butyldioxy)-3,3,5-trimethlcyclohexane on Cultured Chinese Hamster Cells

要約

1,1-ビス(*tert*-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサンの染色体異常誘発性の有無を検討するため，チャイニーズ・ハムスター肺由来の線維芽細胞株(CHL/IU)を用いて*in vitro*における染色体異常試験を実施した．

染色体異常試験に用いる用量を決定するため，細胞増殖抑制試験を行った結果，短時間処理法S9 mix非存在下では500 μg/mLでのみ50 %をやや上回る細胞増殖抑制が認められたが，その他の用量では50 %を上回る細胞増殖抑制は認められなかった．S9 mix存在下では50 %を上回る細胞増殖抑制は認められなかった．連続処理法24時間処理では50 μg/mL以上で50 %を上回る細胞増殖抑制が認められた．したがって，染色体異常試験における用量は，短時間処理法の場合，S9 mix非存在下では93.75，187.5，375，750，1500および3000 μg/mL，S9 mix存在下では375，750，1500および3000 μg/mLとした．また，連続処理法24時間処理では6.25，12.5，25，37.5，50および100 μg/mLとした．

試験の結果，短時間処理法S9 mix非存在および存在下並びに連続処理法24時間処理のいずれの場合においても，染色体異常を有する細胞の増加は認められなかった．

以上の成績から，1,1-ビス(*tert*-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサンのCHL/IU細胞に対する染色体異常誘発性は陰性と判定した．

方法

1. 試験細胞株

国立医薬品食品衛生研究所変異遺伝部(元：国立衛生試験所変異原性部)から昭和60年1月13日に分与を受けたチャイニーズ・ハムスター肺由来の線維芽細胞株(CHL/IU)を使用した．供試細胞は，浮遊細胞液に10 vol%の割合でジメチルスルホキシド(DMSO，和光純薬工業㈱)を添加し，液体窒素条件下で保存したものを培養液に戻し，解凍後の継代数が7回までのものを使用した．

2. 培養液

Eagle-MEM粉末培地(Gibco Laboratories)を常法に従い調製し，これに非働化仔牛血清(Gibco Laboratories)を10 vol%の割合で添加したものを用いた．

3. 培養条件

4×10^3個/mLの細胞を含む培養液5 mLをディッシュ(径6 cm, Becton Dickinson Co.)に加え，37 ℃のCO_2インキュベーター(5 % CO_2)内で培養した．

短時間処理法では，培養開始3日後に被験物質を加えS9 mix非存在および存在下で6時間処理し，処理終了後，新鮮培養液でさらに18時間培養した．連続処理法では，培養開始3日後に被験物質を加え24時間処理した．

4. S9 mix

染色体異常試験用凍結S9 mix(キッコーマン㈱)を購入し，製造後6ヶ月以内に使用した．S9は，誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawley系雄ラットの肝臓から調製されたものである．

5. 被験物質

1,1-ビス(*tert*-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサン(ロット番号8X01，日本油脂㈱(東京)提供)は，無色ないし淡黄色透明の液体で，水およびDMSOに不溶，アセトンに易溶であり，純度97.9 %(不純物として，原料である3,3,5-トリメチルシクロヘキサン約1.89 %を含む)の物質である．被験物質は，冷暗所(4 ℃)で密栓保管した．

実験終了後，残余被験物質を分析した結果，安定性に問題はなかった．

6. 被験物質供試液の調製

溶媒にアセトン(和光純薬工業㈱)を用い，被験物質を溶解して最高用量の供試液(原液)を調製した．この原液の一部を溶媒で順次希釈して所定用量の供試液を調製した．供試液は，用時調製し，そのディッシュ内への添加量は培養液量の0.5 vol%とした．

7. 細胞増殖抑制試験

染色体異常試験に用いる被験物質の用量を決定するため，被験物質の細胞増殖に及ぼす影響を調べた．0.1 w/v%クリスタルバイオレット水溶液で染色した細胞の密度を単層培養細胞密度計(モノセレーターII，MI-60，オリンパス光学工業㈱)を用いて測定し，溶媒対照群の細胞増殖率を100 %とした時の各用量群の細胞増殖率を求めた．

その結果(Fig. 1, 2)，短時間処理法においては，S9 mix非存在下では500 μg/mLで50 %をやや上回る細胞増

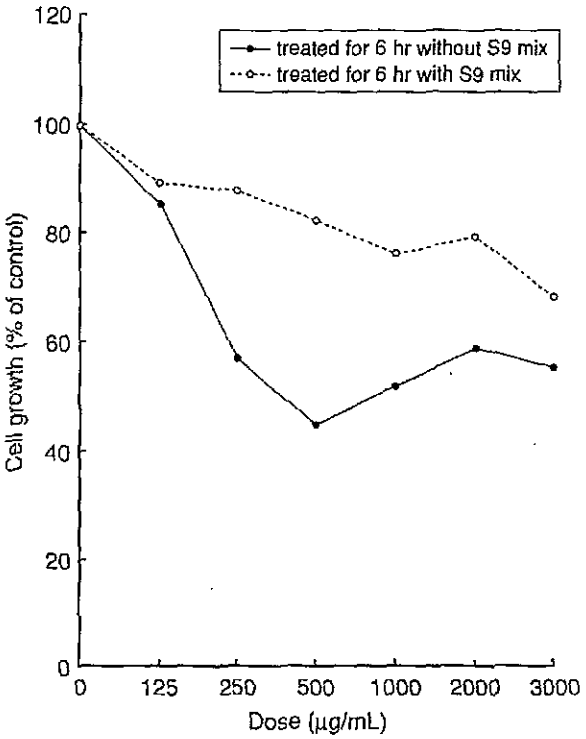

Fig. 1 Growth inhibition of CHL/IU cells treated with 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane

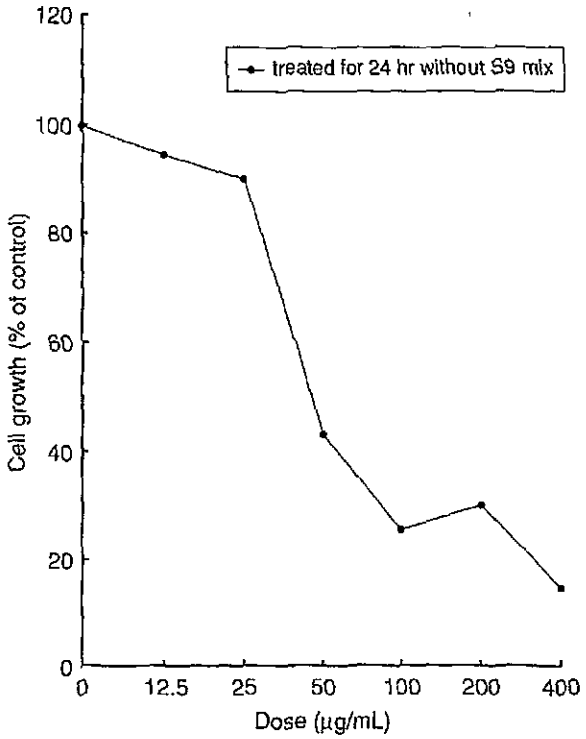

Fig. 2 Growth inhibition of CHL/IU cells treated with 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane

殖抑制が認められたものの，他の用量においては50 %を上回る細胞増殖抑制は認められなかった．また，S9 mix存在下ではいずれの用量においても50 %を上回る細胞増殖抑制は認められず，50 %細胞増殖抑制用量は3000 μg/mL以上と判断された．連続処理法24時間処理では50 μg/mL以上で50 %を上回る細胞増殖抑制が認められ，50 %細胞増殖抑制用量は25~50 μg/mLの用量域にあるものと判断された．

8. 実験群の設定

細胞増殖抑制試験の結果から，染色体異常試験における被験物質の用量は，短時間処理法の場合は，10 mMに相当する3000 μg/mLを最高用量とし，S9 mix非存在下では93.75，187.5，375，750，1500および3000 μg/mLの6用量(公比2)，S9 mix存在下では375，750，1500および3000 μg/mLの4用量(公比2)とした．連続処理法24時間処理の場合は，50 %細胞増殖抑制用量の前後が含まれ，かつ3用量以上のデータが得られることを考慮して，6.25，12.5，25，37.5，50および100 μg/mLとした．対照として，溶媒対照群と陽性対照群を設けた．

陽性対照として，短時間処理法S9 mix存在下では3,4-benzo[*a*]pyrene(B[*a*]P, Sigma Chemical Co.)を10 μg/mL，短時間処理法S9 mix非存在下および連続処理法では1-methyl-3-nitro-1-nitrosoguanidine(MNNG, Aldrich Chemical Co.)を2.5 μg/mLの用量で用いた．陽性対照物質の溶媒には，いずれもDMSO(和光純薬工業㈱)を使用した．

9. 染色体標本の作製

培養終了2時間前にコルセミド(Gibco Laboratories)を最終濃度として0.2 μg/mLとなるように添加した．トリプシン処理で細胞を剥離し，遠心分離により細胞を回収した．75 mM塩化カリウム水溶液で低張処理後，用時調製した冷却メタノール・酢酸(3:1)混合液で細胞を固定した．空気乾燥法で染色体標本を作製した後，1.4 vol%ギムザ液で約15分間染色した．

10. 染色体の観察

各ディッシュあたり100個，すなわち，1用量当たり2ディッシュ，200個の分裂中期像を，総合倍率600倍の顕微鏡下で観察した．標本は全てコード化し，盲検法で観察を行った．染色体の分析は，日本環境変異原学会・哺乳動物試験分科会(MMS)による分類法[1]に基づいて行い，染色体型あるいは染色分体型の切断，交換などの構造異常と倍数性細胞(Polyploid)の有無について観察した．

11. 記録と判定

観察した細胞数，構造異常の種類と数および倍数性細胞の数について集計し，記録した．

染色体構造異常細胞および倍数性細胞の出現頻度について，多試料χ^2検定を行い有意差(有意水準5 %以下)が認められた場合は，フィッシャーの直接確率法を用いて溶媒対照群と各用量群との間の有意差検定(有意水準は多重性を考慮して，5 %または1 %を処理群の数で割ったものを用いた)を行った．

その結果，溶媒対照群と比較して，被験物質による染色体異常細胞の出現頻度が2用量以上で有意に増加し，かつ用量依存性あるいは再現性が認められた場合，陽性と判定した．

結果および考察

短時間処理法による結果をTable 1に示す．1,1-ビス(*tert*-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサンを加えてS9 mix非存在および存在下で6時間処理したいずれの用量群においても，染色体の構造異常および倍数性細胞の誘発作用は認められなかった．

連続処理法による結果をTable 2に示す．被験物質を加えて24時間処理したいずれの用量においても，染色体の構造異常および倍数性細胞の誘発作用は認められなかった．

したがって，1,1-ビス(*tert*-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサンのCHL/IU細胞に対する染色体異常誘発性は陰性と判定した．本試験結果は，CHL/IU細胞において，染色体異常を有する細胞の出現頻度が5%未満を陰性とする石館らの判定基準[2]からみても，明らかに陰性と判断されるものであった．

なお，1,1-ビス(*tert*-ブチルジオキシ)-3,3,5-トリメチルシクロヘキサンおよびその類縁化合物の変異原性に関する報告は見当らない．

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Table 1 Chromosome analysis of Chinese hamster cells (CHL/IU) treated with 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane with and without S9 mix

Group	Dose (μg/mL)	S9 mix	Time of exposure (hr)	No. of cells analysed	No. of cells with structural aberrations						gap (%)	No. of cells with numerical aberrations		Cell Growth rate (%)
					ctb	cte	csb	cse	oth	total (%)[2]		Polyploid	total (%)[2]	
Solvent[1]	0	-	6-(18)	200	1	0	0	2	0	3(1.5)	0(0)	0	0(0)	100.0
BTBTC	93.75	-	6-(18)	200	0	0	0	1	0	1(0.5)	0(0)	0	0(0)	70.5
	187.5	-	6-(18)	200	2	0	0	0	0	2(1.0)	0(0)	0	0(0)	50.0
	375	-	6-(18)	200	0	0	0	0	0	0(0)	1(0.5)	0	0(0)	50.5
	750	-	6-(18)	200	0	1	0	0	0	1(0.5)	1(0.5)	0	0(0)	41.5
	1500	-	6-(18)	200	0	1	0	0	0	1(0.5)	0(0)	1	1(0.5)	41.0
	3000	-	6-(18)	200	0	1	0	2	0	2(1.0)	0(0)	1	1(0.5)	46.5
MNNG	2.5	-	6-(18)	200	64	194	1	0	0	196(98.0)**	4(2.0)	0	0(0)	—
Solvent[1]	0	+	6-(18)	200	0	1	0	1	0	2(1.0)	0(0)	0	0(0)	100.0
BTBTC	375	+	6-(18)	200	0	0	0	1	0	1(0.5)	0(0)	0	0(0)	97.5
	750	+	6-(18)	200	0	0	0	0	0	0(0)	0(0)	1	1(0.5)	102.0
	1500	+	6-(18)	200	0	0	0	0	0	0(0)	0(0)	0	0(0)	98.5
	3000	+	6-(18)	200	0	0	0	0	0	0(0)	2(1.0)	0	0(0)	90.0
BP	10	+	6-(18)	200	12	90	0	0	0	93(46.5)**	1(0.5)	0	0(0)	—

Abbreviations; gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring), oth: others, SA: structural aberration, NA: numerical aberration, BTBTC: 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane, MNNG: 1-methyl-3-nitro-1-nitrosoguanidine, BP: 3,*A*-benzo[a]pyrene
1) Acetone was used as solvent. 2) Multi-sample χ^2 test was done at $p<0.05$ and then Fisher's exact test was done at $p<0.05$ or $p<0.01$.
**: Significantly different from solvent group data at $p<0.01$ by Fisher's exact test.

Table 2 Chromosome analysis of Chinese hamster cells (CHL/IU) continuously treated with 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane without S9 mix

Group	Dose (μg/mL)	Time of exposure (hr)	No. of cells analysed	No. of cells with structural aberrations						gap (%)	No. of cells with numerical aberrations		Cell Growth rate (%)
				ctb	cte	csb	cse	oth	total (%)[2]		Polyploid	total (%)[2]	
Solvent[1]	0	24	200	0	0	0	0	0	0(0)	2(1.0)	2	2(1.0)	100.0
BTBTC	6.25	24	200	0	0	0	0	0	0(0)	1(0.5)	0	0(0)	91.5
	12.5	24	200	0	0	0	0	0	0(0)	0(0)	2	2(1.0)	86.5
	25	24	200	1	1	0	0	0	1(0.5)	1(0.5)	0	0(0)	85.0
	37.5	24	200	0	2	0	0	0	2(1.0)	1(0.5)	1	1(0.5)	70.5
	50	24	200	0	1	0	1	0	2(1.0)	0(0)	0	0(0)	52.5
	100	24	200	0	1	0	0	0	1(0.5)	0(0)	1	1(0.5)	34.0
MNNG	2.5	24	200	60	194	3	0	0	195(97.5)**	0(0)	0	0(0)	—

Abbreviations; gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring), oth: others, SA: structural aberration, NA: numerical aberration, BTBTC: 1,1-bis(*tert*-butyldioxy)-3,3,5-trimethylcyclohexane, MNNG: 1-methyl-3-nitro-1-nitrosoguanidine, BP: 3,*A*-benzo[a]pyrene
1) Acetone was used as solvent. 2) Multi-sample χ^2 test was done at $p<0.05$ and then Fisher's exact test was done at $p<0.05$ or $p<0.01$.
**: Significantly different from solvent group data at $p<0.01$ by Fisher's exact test.

Fd Chem. Toxic. Vol. 31, No. 12, pp. 929–933, 1993
Printed in Great Britain.

0278-6915/93 $6.00 + 0.00

Research Section

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

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(Accepted 14 July 1993)

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

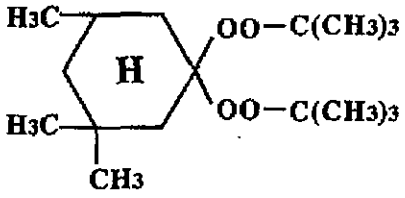

Fig. 1. Chemical structure of 1,1-bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane (BBTC).

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

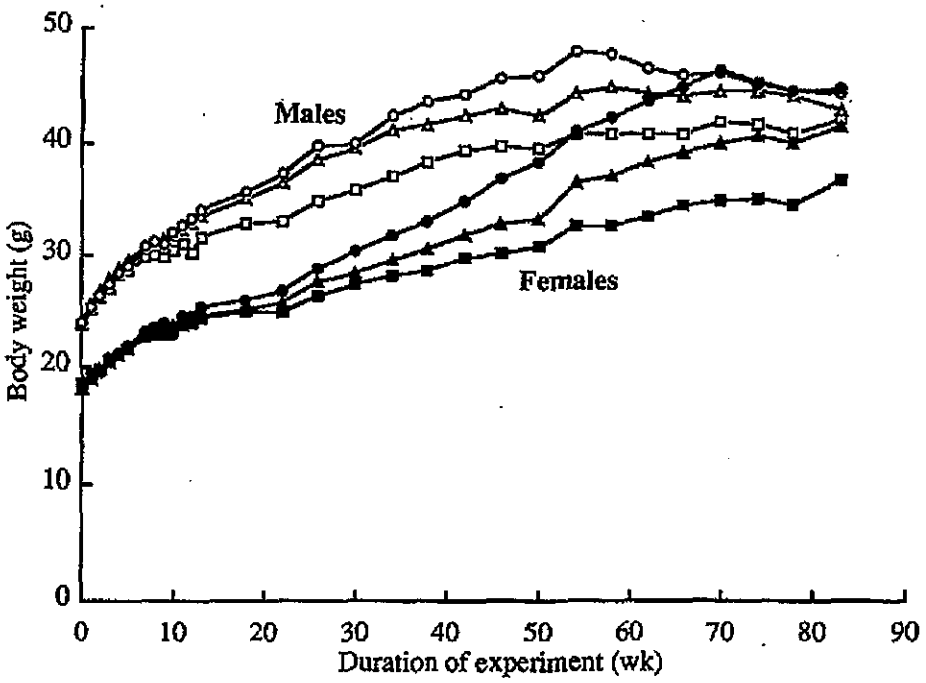

Fig. 2. Growth curves of B6C3F$_1$ mice given 1,1-bis(*tert*-butylperoxy)-3,3,5-trimethylcyclohexane in the diet for 78 wk at 0 (males, ○; females, ●), 0.25 (males, △; females, ▲) or 0.5% (males, □; females, ■). Surviving mice were observed for a further 10 wk and killed at 83 wk.

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-trimethylcyclohexane (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	Males			Females		
BBTC dose (%)	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.

Acknowledgement—This work was supported by a Grant-in-Aid for Safety Evaluation of Existing Chemicals from the Ministry of Health and Welfare of Japan.

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