Table 1
Mutant frequencies induced by BfQ, BhQ and 1,7-Phe in five organs of Muta™Mouse for the expression time of 14 days

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5	No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5
Liver	Control (olive oil)	1120000	106	9.5	7.0 ± 1.6	449400	12	2.7	2.1 ± 0.6
		816000	59	7.2		938400	24	2.6	
		1198000	73	6.1		764400	11	1.4	
		791500	41	5.2		699000	11	1.6	
	BfQ	634500	32	5.0	9.6 ± 3.1	804600	12	1.5	3.8 ± 1.6
		590000	51	8.6		662400	35	5.3	
		158500	20	12.6		426300	23	5.4	
		221500	27	12.2		883200	28	3.2	
	BhQ	442000	29	6.6	5.9 ± 1.1	1188000	53	4.5	2.6 ± 1.1
		677000	39	5.8		1134600	21	1.9	
		257500	11	4.3		671700	17	2.5	
		645500	46	7.1		1011000	15	1.5	
	1,7-Phe	272000	41	15.1	15.9 ± 0.5**	813600	44	5.4	4.1 ± 0.8*
		183000	30	16.4		562800	19	3.4	
		263000	43	16.3		720000	25	3.5	
		184000	29	15.8		606000	25	4.1	
Spleen	Control (olive oil)	855500	116	13.6	7.3 ± 3.6	623100	12	1.9	2.4 ± 0.3
		533000	29	5.4		1502400	41	2.7	
		446500	25	5.6		546900	15	2.7	
		461000	22	4.8		569400	13	2.3	
	BfQ	210500	13	6.2	6.1 ± 0.6	2098200	25	1.2	3.5 ± 2.8
		244500	16	6.5		441000	13	2.9	
		403000	27	6.7		785400	65	8.3	
		256500	13	5.1		786600	13	1.7	
	BhQ	297000	12	4.0	6.5 ± 1.6	277800	10	3.6	2.9 ± 0.6
		354500	25	7.1		828300	22	2.7	
		396500	26	6.6		946200	31	3.3	
		544000	46	8.5		1608600	34	2.1	
	1,7-Phe	426500	34	8.0	7.0 ± 1.0	967200	20	2.1	2.4 ± 0.7
		502500	27	5.4		1023000	24	2.3	
		320000	24	7.5		1026900	16	1.6	
		462500	34	7.4		905400	32	3.5	
Lung	Control (olive oil)	1539500	127	8.2	6.0 ± 1.3	1027800	21	2.0	2.1 ± 0.4
		1111500	60	5.4		738000	21	2.8	
		678000	35	5.2		1142700	20	1.8	
		1473000	76	5.2		831600	15	1.8	
	BfQ	553000	39	7.1	6.0 ± 0.6	1107600	18	1.6	2.6 ± 0.6
		332000	18	5.4		903300	22	2.4	
		353000	21	5.9		1124700	36	3.2	
		266000	15	5.6		445200	14	3.1	
	BhQ	401500	51	12.7	10.8 ± 2.4*	1705500	37	2.2	3.5 ± 1.0
		481500	54	11.2		1071000	33	3.1	
		572500	72	12.6		2403000	99	4.1	
		372000	25	6.7		2083200	98	4.7	

Table 1 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5	No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5
	1,7-Phe	335500	29	8.6	10.3 ± 1.9*	1103400	26	2.4	2.9 ± 0.5
		351000	46	13.1		1012200	27	2.7	
		244500	27	11.0		909600	34	3.7	
		211000	18	8.5		892200	26	2.9	
Kidney	Control (olive oil)	219500	15	6.8	6.8 ± 1.4	551100	21	3.8	2.7 ± 1.0
		190000	17	8.9		426600	16	3.8	
		349500	17	4.9		588000	11	1.9	
		301000	20	6.6		771000	12	1.6	
	BfQ	682500	46	6.7	7.4 ± 1.1	1035000	25	2.4	3.9 ± 0.9
		550500	51	9.3		825000	36	4.4	
		474000	33	7.0		649800	30	4.6	
		484000	32	6.6		1599000	66	4.1	
	BhQ	920500	55	6.0	7.4 ± 1.2	1323600	26	2.0	2.2 ± 0.5
		622000	51	8.2		945000	27	2.9	
		113000	10	8.8		1408800	23	1.6	
		244500	16	6.5		1018200	23	2.3	
	1,7-Phe	486500	30	6.2	6.8 ± 0.5	814800	15	1.8	3.3 ± 1.6
		558000	38	6.8		660300	40	6.1	
		177000	12	6.8		520200	13	2.5	
		319500	24	7.5		1664700	48	2.9	
Bone marrow	Control (olive oil)	311000	32	10.3	7.1 ± 3.0	644100	14	2.2	1.3 ± 0.6
		465000	27	5.8		1041000	16	1.5	
		70500	2	2.8		111300	1	0.9	
		96500	9	9.3		154500	1	0.6	
	BfQ	325500	17	5.2	6.4 ± 0.9	1075200	16	1.5	2.6 ± 0.9
		256500	17	6.6		528000	12	2.3	
		326000	25	7.7		572100	22	3.8	
		708500	44	6.2		1144800	32	2.8	
	BhQ	257000	13	5.1	5.7 ± 0.5	1757100	20	1.1	1.7 ± 0.4
		617000	38	6.2		1349400	24	1.8	
		683000	41	6.0		1040400	22	2.1	
	1,7-Phe	502500	24	4.8	4.7 ± 0.5	963600	12	1.2	1.6 ± 0.6
		397500	19	4.8		962400	11	1.1	
		622000	33	5.3		1341000	21	1.6	
		332000	13	3.9		916900	24	2.6	

* Significantly different from the control group, $P < 0.05$.
** Significantly different from the control group, $P < 0.01$.

selection for *cII* mutants was performed according to the method of Jakubczak et al. [24] with slight modification as previously reported [14]. Briefly, the phage solution was absorbed to *E. coli* G1225 (*hfl*$^-$) at room temperature for 20–30 min. For titration, an appropriately diluted phage-*E. coli* solution was mixed with LB top agar (containing 10 mM $MgSO_4$), plated onto dishes containing bottom agar, and incubated for 24 h at 37 °C. The remaining phage-*E. coli* solution was mixed with LB top agar and plated onto dishes con-

Table 2
Mutant frequencies induced by BfQ and 1,7-Phe in five organs of Muta™Mouse for the expression time of 56 days

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5	No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5
Liver	Control (olive oil)	246500	21	8.5	7.9 ± 1.3	960700	18	1.9	1.7 ± 0.2
		168500	12	7.1		1161000	21	1.8	
		636500	39	6.1		3351900	65	1.9	
		155500	15	9.6		1978900	27	1.4	
	BfQ	259000	24	9.3	11.5 ± 2.8	543600	14	2.6	2.4 ± 0.2[a]
		367000	35	9.5		2746500	60	2.2	
		714000	116	16.2		3693000	97	2.6	
		180500	20	11.1		2490600	57	2.3	
	1,7-Phe	653000	63	9.6	14.8 ± 3.7[a]	1468200	64	4.4	4.8 ± 1.2[a]
		266500	35	13.1		1140000	36	3.2	
		497000	94	18.9		4469100	286	6.4	
		126000	22	17.5		306600	16	5.2	
Spleen	Control (olive oil)	608500	47	7.7	7.8 ± 0.4	1825800	53	2.9	2.9 ± 0.6
		347500	26	7.5		1304400	48	3.7	
		355500	30	8.4		1224600	36	2.9	
		389500	29	7.4		1106400	22	2.0	
	BfQ	440000	38	8.6	8.4 ± 0.3	2245800	32	1.4	3.3 ± 2.5
		242500	21	8.7		860400	14	1.6	
		354000	30	8.5		1090800	83	7.6	
		460500	36	7.8		946200	24	2.5	
	1,7-Phe	567000	81	14.3	10.0 ± 2.5	1022400	22	2.2	2.2 ± 0.04
		231500	18	7.8		976800	22	2.3	
		336000	29	8.6		1059000	23	2.2	
		253500	24	9.5		865800	19	2.2	
Lung	Control (olive oil)	390500	25	6.4	7.9 ± 1.9	657600	13	2.0	3.1 ± 0.9
		218500	21	9.6		1230600	30	2.4	
		558500	32	5.7		936000	35	3.7	
		474500	47	9.9		928800	38	4.1	
	BfQ	332500	26	7.8	7.2 ± 1.2	742200	19	2.6	3.7 ± 1.9
		554500	43	7.8		1035000	25	2.4	
		476000	39	8.2		839400	59	7.0	
		386000	20	5.2		651000	17	2.6	
	1,7-Phe	731500	56	7.7	7.7 ± 0.9	1365600	25	1.8	3.0 ± 0.9
		412000	32	7.8		728400	29	4.0	
		494500	44	8.9		966600	26	2.7	
		519000	33	6.4		946800	35	3.7	
Kidney	Control (olive oil)	442500	26	5.9	8.2 ± 2.4	1874400	64	3.4	2.5 ± 0.6
		217000	26	12.0		2313600	43	1.9	
		383000	25	6.5		1139400	25	2.2	
		596500	50	8.4		1437600	39	2.7	
	BfQ	552500	41	7.4	7.1 ± 1.0	1629600	60	3.7	2.9 ± 0.7
		479000	39	8.1		1360800	33	2.4	
		774500	56	7.2		1446000	50	3.5	
		698500	38	5.4		1277400	27	2.1	

Table 2 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5	No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5
	1,7-Phe	299500	19	6.3	7.3 ± 1.1	1201800	26	2.2	2.2 ± 0.3
		567500	50	8.8		1393200	35	2.5	
		877500	70	8.0		1409400	35	2.5	
		513500	32	6.2		1060200	19	1.8	
Bone marrow	Control (olive oil)	607500	43	7.1	7.9 ± 1.2	1334700	21	1.6	1.9 ± 0.4
		829000	57	6.9		1204800	25	2.1	
		924500	70	7.6		1441800	23	1.6	
		605500	60	9.9		1184400	30	2.5	
	BfQ	429000	26	6.1	6.9 ± 2.1	1703400	19	1.1	3.9 ± 4.1
		661500	69	10.4		1287600	15	1.2	
		893000	47	5.3		1256400	136	10.8	
		791500	45	5.7		1249200	29	2.3	
	1,7-Phe	605500	92	15.2	9.0 ± 3.8	1206600	13	1.1	1.5 ± 0.3
		447500	39	8.7		1700400	22	1.3	
		507000	30	5.9		876000	14	1.6	
		1188000	71	6.0		1444200	28	1.9	

* Significantly different from the control group, $P < 0.05$.
** Significantly different from the control group, $P < 0.01$.

taining bottom agar. The plates were incubated for 48 h at 25 °C for selection of *cII* mutants. The wild type phage, recovered from Muta™Mouse, has a *cI*⁻ phenotype, which permits plaque formation with the *hfl*⁻ strain at 37 °C but not at 25 °C. The mutant frequency was calculated by the following formula:

$$\text{mutant frequency} = \left(\frac{\text{total number of plaques on selection plates}}{\text{total number of plaques on titer plates}}\right) \times \text{dilution factor.}$$

The significance of differences in the mutant frequency between the treated and control groups was analyzed by using Student's *t*-test.

2.2.5. Sequencing of mutants

The entire lambda *cII* region was amplified directly from mutant plaques by the use of Taq DNA polymerase (Takara Shuzo, Tokyo, Japan) with primers P1; 5′-AAAAAGGGCATCAAATTAAACC-3′, and P2; 5′-CCGAAGTTGAGTATTTTTGCTGT-3′ as previously reported [14] (Fig. 3). A 446 bp PCR product was purified with a microspin column (Amersham Pharmacia, Tokyo, Japan) and then used for a sequencing reaction with the Ampli Taq cycle sequencing kit (PE Biosystems, Tokyo, Japan) using the primer P1. The reaction product was purified by ethanol precipitation and analyzed with the ABI PRISM™ 310 genetic analyzer (PE Biosystems).

3. Results

3.1. Mutant frequency of BfQ, BhQ, and 1,7-Phe

BfQ, BhQ, and 1,7-Phe (Fig. 1) were tested for in vivo mutagenicity using *lacZ* transgenic mice (Muta™Mice). The mutant frequencies observed in the DNA preparations extracted from the five organs are shown in Tables 1 and 2. Over 10 mutant plaques were analyzed in most organs. For the bone marrow in Table 1, the mutant frequency of one animal in the BhQ-treated group was missing and the number of mutants in two animals in the control group was insufficient because the isolated DNA was not enough

to be analyzed. The spontaneous mutant frequencies observed in the control group were similar for the five organs in both *lacZ* and *cII* assays regardless of the expression time (14 or 56 days), the rate ranging from 6.0 to 8.2 × 10^{-5} and from 1.3 to 3.1 × 10^{-5}, respectively. These results were similar to those of our previous studies [9,12–14].

Table 1 shows mutant frequencies with the test compounds in the five organs 14 days after the last injection. BfQ slightly, but not significantly, increased the mutant frequency in the liver in both assays. On the other hand, BhQ significantly increased the mutant frequency in the lung in the *lacZ* assay. 1,7-Phe significantly increased the mutant frequency in the liver in both assays and in the lung in the *lacZ* assay.

Mutant frequencies observed in the DNA preparations extracted from the five organs 56 days after the last injection are shown in Table 2. BfQ significantly increased the mutant frequency in the liver in the *cII* assay, whereas the mutant frequency in the *lacZ* assay was slightly, but not significantly, increased. 1,7-Phe significantly increased the mutant frequency in the liver in both assays like the results obtained 14 days

Table 3
Sequences of *cII* mutations in the liver of BfQ-treated Muta™Mouse for the expression time of 14 days

Mutant no.	Position	Mutation	Sequence	Amino acid change
A1	113	C to T	AAG TCG CAG	Ser to Leu
A2	99–100	GG to TT	GTG GGC GTT	Gly to Cys
A3	107	A to C	GTT GAT AAG	Asp to Ala
A4	57	C to G	CTT AAC AAA	Asn to Lys
A5	214	C to T	GCG CGA CAA	Arg to Stop
A6	181	G to T	TGG GGG GTC	Gly to Trp
A7	34	C to T	CTA CGA ATC	Arg to Stop
A8	103	G to A	GGC GTT GAT	Val to Ile
A9	196	G to T	GAC GAC ATG	Asp to Tyr
A10	129	G to C	AGG TGG AAG	Trp to Cys
A11	34	C to T	CTA CGA ATC	Arg to Stop
A12	25	G to A	AAC GAG GCT	Glu to Lys
A13	241–246	−A	AAA AAA CGC	Frameshift
A14	179–184	−G	TGG GGG GTC	Frameshift
A15	57	C to A	CTT AAC AAA	Asn to Lys
A16	35	G to T	CTA CGA ATC	Arg to Leu
A17	179–184	+G	TGG GGG GTC	Frameshift
A18	90–91	GG to TT	GCG GAA GCT	Glu to Stop
A19	94	G to T	GAA GCT GTG	Ala to Ser
A20	115	C to T	TCG CAG ATC	Gln to Stop
A21	193	G to A	GAC GAC GAC	Asp to Asn
A22	64	G to A	ATC GCA ATG	Ala to Thr
A23	103	G to A	GGC GTT GAT	Val to Ile
A24	104	T to C	GGC GTT GAT	Val to Ala
A25	89	C to T	ACA GCG GAA	Ala to Val
A26[a]	64	G to A	ATC GCA ATG	Ala to Thr
A27	175	G to T	CTT GAA TGG	Glu to Stop
A28	25	G to A	AAC GAG GCT	Glu to Lys
A29	34	C to T	CTA CGA ATC	Arg to Stop
A30	100	G to A	GTG GGC GTT	Gly to Ser
A31	62	T to C	AAA ATC GCA	Ile to Thr
A32[a]	25	G to A	AAC GAG GCT	Glu to Lys
A33	196	G to A	GAC GAC ATG	Asp to Asn
A34	179–184	−G	TGG GGG GTC	Frameshift
A35	115	C to A	TCG CAG ATC	Gln to Lys
A36	134	G to C	AAG AGG GAC	Arg to Thr

[a] Ascribable to the same mutation obtained in an identical mouse.

after the last injection. 1,7-Phe did not increase the mutant frequency in the lung for the expression time of 56 days.

3.2. Mutation spectra of BfQ, BhQ, and 1,7-Phe-induced mutations

A total of 36 BfQ-induced mutants in the liver for the expression time of 14 days, 37 BhQ-induced mutants in the lung for 14 days, and 43 1,7-Phe-induced mutants in the liver for 56 days were subjected to sequence analysis. The mutations are characterized in Tables 3–5, and summarized in Table 6. In Table 6, the same mutations in an identical mouse were treated as single events. The data of the spontaneous mutations are from our previous report [9].

1,7-Phe-induced mutations consisted mainly of base substitutions (36/39); G:C to A:T transitions (15/39) and G:C to C:G transversions (10/39) predominated. Compared with the spontaneous mutation spectrum, G:C to A:T transitions decreased and G:C to C:G transversions increased in the mutations by

Table 4
Sequences of *cII* mutations in the lung of BhQ-treated Muta™Mouse for the expression time of 14 days

Mutant no.	Position	Mutation	Sequence	Amino acid change
B1	196	G to A	GAC GAC ATG	Asp to Asn
B2	179–184	+G	TGG GGG GTC	Frameshift
B3	149	A to T	CCA AAG TTC	Lys to Met
B4	241–246	−A	AAA AAA CGC	Frameshift
B5	34	C to T	CTA CGA ATC	Arg to Stop
B6	113	C to T	AAG TCG CAG	Ser to Leu
B7	215	G to T	GCG CGA CAA	Arg to Leu
B8[a]	34	C to T	CTA CGA ATC	Arg to Stop
B9	166	G to C	CTT GCT GTT	Ala to Pro
B10	25	G to A	AAC GAG GCT	Glu to Lys
B11	34	C to T	CTA CGA ATC	Arg to Stop
B12	62	T to C	AAA ATC GCA	Ile to Thr
B13[a]	34	C to T	CTA CGA ATC	Arg to Stop
B14	233	T to C	ATT CTC ACC	Leu to Pro
B15	40	G to A	ATC GAG AGT	Glu to Lys
B16	212	C to T	TTG GCG CGA	Ala to Val
B17[a]	212	C to T	TTG GCG CGA	Ala to Val
B18	113	C to T	AAG TCG CAG	Ser to Leu
B19	46	G to C	AGT GCG TTG	Ala to Pro
B20	179–184	+G	TGG GGG GTC	Frameshift
B21	89	C to T	ACA GCG GAA	Ala to Val
B22	196	G to A	GAC GAC ATG	Asp to Asn
B23	190–198	−GAC	GAC GAC GAC	Frameshift
B24	34	C to T	CTA CGA ATC	Arg to Stop
B25	205	C to T	GCT CGA TTG	Arg to Stop
B26	179–184	−G	TGG GGG GTC	Frameshift
B27	122	G to T	ATC AGC AGG	Ser to Ile
B28	28	G to A	GAG GCT CTA	Ala to Thr
B29	52	C to G	TTG CTT AAC	Leu to Val
B30	197	A to G	GAC GAC ATG	Asp to Gly
B31	212	C to T	TTG GCG CGA	Ala to Val
B32	91	G to T	GCG GAA GCT	Glu to Stop
B33	205	C to T	GCT CGA TTG	Arg to Stop
B34	40	G to A	ATC GAG AGT	Glu to Lys
B35	34	C to T	CTA CGA ATC	Arg to Stop
B36[a]	40	G to A	ATC GAG AGT	Glu to Lys
B37	89	C to T	ACA GCG GAA	Ala to Val

[a] Ascribable to the same mutation obtained in an identical mouse.

Table 5
Sequences of *cII* mutations in the liver of 1,7-Phe-treated Muta™Mouse for the expression time of 56 days

Mutant no.	Position	Mutation	Sequence	Amino acid change
C1	113	C to T	AAG TCG CAG	Ser to Leu
C2	212	C to T	TTG GCG CGA	Ala to Val
C3	125	G to C	AGC AGG TGG	Arg to Thr
C4	196	G to A	GAC GAC ATG	Asp to Asn
C5	40	G to A	ATC GAG AGT	Glu to Lys
C6[a]	212	C to T	TTG GCG CGA	Ala to Val
C7	46	G to C	AGT GCG TTG	Ala to Pro
C8	94	G to C	GAA GCT GTG	Ala to Pro
C9	134	G to T	AAG AGG GAC	Arg to Met
C10	163	C to T	CGG CTT GCT	Leu to Phe
C11	34	C to T	CTA CGA ATC	Arg to Stop
C12	179–240	−62 bp		Frameshift
C13	193	G to A	GAC GAC GAC	Asp to Asn
C14	65	C to T	ATC GCA ATG	Ala to Val
C15	164–165	−T	CTT GCT GTT	Frameshift
	166	G to A		
C16	1	A to G	cat ATG GTT	Met to Val
C17	224	C to A	GTT GCT GCG	Ala to Asp
C18	196	G to A	GAC GAC ATG	Asp to Asn
C19	150	G to T	CCA AAG TTC	Lys to Asn
C20	113	C to T	AAG TCG CAG	Ser to Leu
C21[a]	150	G to T	CCA AAG TTC	Lys to Asn
C22	129	G to A	AGG TGG AAG	Trp to Stop
C23	37	A to T	CGA ATC GAG	Ile to Phe
C24	140–141	GG to CT	GAC TGG ATT	Trp to Ser
C25	89	C to A	ACA GCG GAA	Ala to Glu
C26	34	C to T	CTA CGA ATC	Arg to Stop
C27	212	C to T	TTG GCG CGA	Ala to Val
C28	233	T to C	ATT CTC ACC	Leu to Pro
C29	28	G to C	GAG GCT CTA	Ala to Pro
C30	95	C to A	GAA GCT GTG	Ala to Asp
C31	89	C to G	ACA GCG GAA	Ala to Gly
C32	100	G to C	GTG GGC GTT	Gly to Arg
C33	25	G to T	AAC GAG GCT	Glu to Stop
C34	39	C to G	CGA ATC GAG	Ile to Met
C35	103	G to C	GGC GTT GAT	Val to Leu
C36	212	C to T	TTG GCG CGA	Ala to Val
C37	64	G to A	ATC GCA ATG	Ala to Thr
C38	193	G to T	GAC GAC GAC	Asp to Tyr
C39	95	C to A	GAA GCT GTG	Ala to Asp
C40	74	G to C	CTT GGA ACT	Gly to Ala
C41	120	C to G	CAG ATC AGC	Ile to Met
C42[a]	39	C to G	CGA ATC GAG	Ile to Met
C43[a]	64	G to A	ATC GCA ATG	Ala to Thr

[a] Ascribable to the same mutation obtained in an identical mouse.

1,7-Phe. On the other hand, BfQ and BhQ-induced *cII* mutant spectra showed no characteristics compared with that of the control and consisted mainly of G:C to A:T transitions (15/34 and 18/33, respectively).

4. Discussion

In this study, we attempted to investigate the in vivo mutagenicity of three tricyclic aza-arenes, BfQ, BhQ, and 1,7-Phe. They were injected daily for 4 days

Table 6
Summary of *cII* mutation spectra in Muta™Mouse

Mutation class	Control[a] (%)	BfQ[b] (%)	BhQ[c] (%)	1,7-Phe[b] (%)
Total	32 (100)	34 (100)	33 (100)	39 (100)
Base substitution	28 (88)	28 (82)	28 (85)	36 (92)
Transitions				
GC to AT	18 (56)	15 (44)	18 (55)	15 (38)
AT to GC	1 (3)	2 (6)	3 (9)	2 (5)
Transversions				
AT to TA	3 (9)	0 (0)	1 (3)	1 (3)
AT to CG	0 (0)	1 (3)	0 (0)	1 (3)
GC to TA	5 (16)	7 (20)	3 (9)	7 (18)
GC to CG	1 (3)	3 (9)	3 (9)	10 (26)
−1 frameshifts	1 (3)	3 (9)	2 (6)	0 (0)
+1 frameshifts	2 (6)	1 (3)	2 (6)	0 (0)
Deletion	0 (0)	0 (0)	1 (3)	1 (3)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	1 (3)	2 (6)	0 (0)	2 (5)

The same mutations from an identical mouse were counted as single events.
[a] The data of the spontaneous mutations are from our previous report [9].
[b] Mutant plaques from the liver.
[c] Mutant plaques from the lung.

into Muta™Mice at the total doses of 400, 400, and 200 mg/kg intraperitoneally, respectively, based on their tolerance doses determined in preliminary tests. Although these aza-analogs of phenanthrene were weak mutagens in Muta™Mouse, different effects on the target organ specificity and mutant spectrum were observed depending on the N-substituted position.

BfQ increased the mutant frequency in the liver for the expression times of both 14 and 56 days. On the other hand, BhQ increased mutagenicity in the lung, but not in the liver. BfQ has a nitrogen atom in the bay-region and BhQ in the non-bay-region. Therefore, the difference in the nitrogen position in the benzoquinoline molecule might alter the target organ. Quinoline has previously shown a potent in vivo mutagenicity in Muta™Mice [12–14]. These results suggest that in vivo mutagenicity is decreased by the benzene-ring fusion on the quinoline moiety. 1,7-Phe significantly increased mutagenicity in the liver for the expression times of both 14 and 56 days and in the lung for the expression time of 14 days. It may be suggested that 1,7-Phe induced mutation both in the liver and lung because 1,7-Phe has a nitrogen atom in both the bay- and non-bay-regions. Our previous data indicated that metabolic activation of these phenanthrene aza-analogs might take place in the pyridine moiety [18] (Fig. 2). LaVoie and co-workers reported that BfQ might be converted to the ultimate form not only by the bay-region mechanism but also by another mechanism [17], supporting our opinion.

With regard to the suitable expression time in the evaluation of in vivo mutagenicity, different tendencies were observed between the mutagenesis of 1,7-Phe in the liver and that in the lung. 1,7-Phe showed similar mutagenicities in the liver after the expression time of both 14 and 56 days. However, in the lung, 1,7-Phe increased the mutant frequency in the lung after the expression time of 14 days, but not after 56 days. Sun and Heddle reported that mutation by ethylnitrosourea in the liver was more firmly established after about 40 days post-treatment than after 20 days [25]. It seems that an appropriate expression time may be necessary to evaluate the in vivo mutagenicity of chemicals in each organ.

1,7-Phe also depressed the G:C to A:T transition and increased the G:C to C:G transversion like quinoline [14], a hepatomutagen possessing the partial structure of 1,7-Phe, compared with the spontaneous mutation spectrum. Therefore it may be suggested that the increase of G:C to C:G transversions might be a common feature of the quinoline-type metabolic activation in aza-arenes.

Although a major question to be answered is how the position of the nitrogen atom is responsible for the differences in mutagenicity between these tricyclic aza-arenes, the present data suggest that the position of the nitrogen atom in the polycyclic aromatic ring might influence in vivo mutagenicity with respect to the target organ specificity and mutational pattern.

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References

[1] I. Schmeltz, D. Hoffmann, Nitrogen-containing compounds in tobacco and tobacco smoke, Chem. Rev. 77 (1977) 295–311.
[2] M. Dong, D.C. Locke, D. Hoffmann, Characterization of aza-arenes in basic organic portion of suspended particulate matter, Environ. Sci. Technol. 11 (1977) 612–618.
[3] C.R. Engel, E. Sawicki, A superior thin-layer chromatographic procedure for the separation of aza arenes and its application to air pollution, J. Chromatogr. 31 (1967) 109–119.
[4] D. Brocco, A. Cimmino, M. Possanzini, Determination of aza-heterocyclic compounds in atmospheric dust by a combination of thin-layer and gas chromatography, J. Chromatogr. 84 (1973) 371–377.
[5] A. Lacassagne, N.P. Buu-Hoï, F. Zajdela, P. Mabille, Carcinogenic activity of some isosteric nitrogenous pentacyclic hydrocarbon carcinogens, Compt. Rend. 258 (1964) 3387–3389.
[6] Y. Kitahara, H. Okuda, K. Shudo, T. Okamoto, M. Nagao, Y. Seino, T. Sugimura, Synthesis and mutagenicity of 10-azabenzo[*a*]pyrene-4,5-oxide and other pentacyclic aza-arene oxides, Chem. Pharm. Bull. (Tokyo) 26 (1978) 1950–1953.
[7] H. Okuda, Y. Kitahara, K. Shudo, T. Okamoto, Identification of an ultimate mutagen of 10-azabenzo[*a*]pyrene: microsomal oxidation of 10-azabenzo[*a*]pyrene to 10-azabenzo[*a*]pyrene-4,5-oxide, Chem. Pharm. Bull. (Tokyo) 27 (1979) 2547–2549.
[8] C.H. Ho, B.R. Clark, M.R. Guerin, B.D. Barkenbus, T.K. Rao, J.L. Epler, Analytical and biological analysis of test materials from the synthetic fuel technologies, Mutat. Res. 85 (1981) 335–345.
[9] K. Yamada, T. Suzuki, A. Kohara, M. Hayashi, A. Hakura, T. Mizutani, K.I. Saeki, Effect of 10-aza-substitution on benzo[*a*]pyrene mutagenicity in both in vivo and in vitro, Mutat. Res. 521 (2002) 187–200.
[10] K. Hirao, Y. Shinohara, H. Tsuda, S. Fukushima, M. Takahashi, Carcinogenic activity of quinoline on rat liver, Cancer Res. 36 (1976) 329–335.
[11] Y. Shinohara, T. Ogiso, M. Hananouchi, K. Nakanishi, T. Yoshimura, N. Ito, Effect of various factors on the induction of liver tumors in animals by quinoline, Jpn. J. Cancer Res. 68 (1977) 785–796.
[12] T. Suzuki, Y. Miyata, K.I. Saeki, Y. Kawazoe, M. Hayashi, T. Sofuni, In vivo mutagenesis by the hepatocarcinogen quinoline in the *lacZ* transgenic mouse: evidence for its in vivo genotoxicity, Mutat. Res. 412 (1998) 161–166.
[13] Y. Miyata, K.I. Saeki, Y. Kawazoe, M. Hayashi, T. Sofuni, T. Suzuki, Antimutagenic structure modification of quinoline assessed by an in vivo mutagenesis assay using *lacZ*-transgenic mice, Mutat. Res. 414 (1998) 165–169.
[14] T. Suzuki, X. Wang, Y. Miyata, K.I. Saeki, A. Kohara, Y. Kawazoe, M. Hayashi, T. Sofuni, Hepatocarcinogen quinoline induces G:C to C:G transversions in the *cII* gene in the liver of lambda/*lacZ* transgenic mice (MutaMouse), Mutat. Res. 456 (2000) 73–81.
[15] R.S. Baker, A.M. Bonin, I. Stupans, G.M. Holder, Comparison of rat and guinea pig as sources of the S9 fraction in the *Salmonella*/mammalian microsome mutagenicity test, Mutat. Res. 71 (1980) 43–52.
[16] G.M. Seixas, B.M. Andon, P.G. Hollingshead, W.G. Thilly, The aza-arenes as mutagens for *Salmonella typhimurium*, Mutat. Res. 102 (1982) 201–212.
[17] S. Kumar, H.C. Sikka, S.K. Dubey, A. Czech, N. Geddie, C.X. Wang, E.J. LaVoie, Mutagenicity and tumorigenicity of dihydrodiols, diol epoxides, and other derivatives of benzo(*f*)quinoline and benzo(*h*)quinoline, Cancer Res. 49 (1989) 20–24.
[18] K.I. Saeki, H. Kawai, Y. Kawazoe, A. Hakura, Dual stimulatory and inhibitory effects of fluorine-substitution on mutagenicity: an extension of the enamine epoxide theory for activation of the quinoline nucleus, Biol. Pharm. Bull. 20 (1997) 646–650.
[19] M. Dong, I. Schmeltz, E. LaVoie, D. Hoffmann, Aza-arenes in the respiratory environment: analysis and assays for mutagenicity, in: P.W. Jones, R.I. Freudenthal (Eds.), Carcinogenesis: A Comprehensive Survey, vol. 3, Raven, New York, 1978, pp. 97–108.
[20] E.A. Adams, E.J. LaVoie, D. Hoffmann, Mutagenicity and metabolism of azaphenanthrenes, in: M.C. Cooke, A.J. Dennis (Eds.), Polynuclear Aromatic Hydrocarbons, Formation, Metabolism, and Measurement, Battelle Press, Columbus, OH, 1983, pp. 73–87.
[21] W.C. Summers, P.M. Glazer, D. Malkevich, Lambda phage shuttle vectors for analysis of mutations in mammalian cells in culture and in transgenic mice, Mutat. Res. 220 (1989) 263–268.
[22] E.J. Gunther, N.E. Murray, P.M. Glazer, High efficiency, restriction-deficient in vitro packaging extracts for bacteriophage lambda DNA using a new *E. coli* lysogen, Nucleic Acids Res. 21 (1993) 3903–3904.

[23] J.A. Gossen, A.C. Molijn, G.R. Douglas, J. Vijg, Application of galactose-sensitive *E. coli* strains as selective hosts for *LacZ*-plasmids, Nucleic Acids Res. 20 (1992) 3254.
[24] J.L. Jakubczak, G. Merlino, J.E. French, W.J. Muller, B. Paul, S. Adhya, S. Garges, Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for in vivo mutations in a bacteriophage lambda transgene target, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 9073–9078.
[25] B. Sun, J.A. Heddle, The relationship between mutant frequency and time in vivo: simple predictions for any tissue, cell type, or mutagen, Mutat. Res. 425 (1999) 179–183.

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Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells

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Abstract

Toxic cyanobacteria (blue-green algae) water blooms have become a serious problem in several industrialized areas of the world. Microcystin-LR (MCLR) is a cyclic heptapeptidic toxin produced by the cyanobacteria. In the present study, we used human lymphoblastoid cell line TK6 to investigate the in vitro genotoxicity of MCLR. In a standard 4 h treatment, MCLR did not induce a significant cytotoxic response at <80 μg/ml. In a prolonged 24 h treatment, in contrast, it induced cytotoxic as well as mutagenic responses concentration-dependently starting at 20 μg/ml. At the maximum concentration (80 μg/ml), the micronucleus frequency and the mutation frequency at the heterozygous thymidine kinase (*TK*) locus were approximately five-times the control values. Molecular analysis of the *TK* mutants revealed that MCLR specifically induced loss of heterozygosity at the *TK* locus, but not point mutations or other small structural changes. These results indicate that MCLR had a clastogenic effect. We discuss the mechanisms of MCLR genotoxicity and the possibility of its being a hepatocarcinogen.

Keywords: Cyanobacteria; Microcystin-LR; Micronucleus test; TK-gene mutation

1. Introduction

Water pollution by cyanobacteria (blue-green algae) causes serious environmental and public health problems in several areas of the world [1–3]. Some genera, such as *Microsystis*, *Oscillatoria*, and *Anabaena* produce microcystines, cyclic heptapeptides, with potent hepatotoxic activity. Fifty different cyanobacterial microcystines have been discovered. They have caused the death of fish, birds, wild animals, and livestock [1,4] and sometimes have had adverse health effects on humans through contaminated residential water supplies [5,6].

Microcystin-LR (MCLR) is the most toxic microcystine. Only 1–2 μg MCLR given intraperitoneally is lethal to mice, with most accumulating in the liver [7,8]. While MCLR hepatotoxicity has been well documented in vitro and in vivo [9–12], few reports describe its genotoxicity. MCLR is not genotoxic in the Ames test, although cyanobacterial extracts are, both with and without metabolic activation [13]. In a human cancer cell line, on the other hand, MCLR induces point mutations, and it produces DNA fragmentation and degradation in mouse liver in vivo [14,15].

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To evaluate the in vitro genotoxicity of MCLR, we used the in vitro micronucleus (MN) assay and the thymidine kinase (*TK*) gene mutation assay on treated human lymphoblatoid TK6 cells [16,17]. The *TK* gene mutation assay is capable of detecting a wide range of genetic damage, including gene mutations, large scale chromosomal changes, recombination, and aneuploidy. Most of the changes occur in human tumors and are presumably relevant to carcinogenesis. Use of a human cell line makes this genotoxicity evaluation appropriate for human hazard evaluation. Molecular analysis of the *TK*-mutants may help us understand the genotoxic mechanism of MCLR [18,19].

2. Materials and methods

2.1. Cells culture and chemical treatment

The TK6 human lymphoblastoid cell line has been described previously [18]. Cells were grown in RPMI1640 medium (Gibco-BRL, Life technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 unit/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated at 37 °C in a 5% CO_2 atmosphere with 100% humidity and maintained at densities ranging from 10^5 to 10^6 cells/ml.

MCLR (Cas.# 101043-37-2) was purchased from Wako Pure Chemical Co. (Tokyo, Japan) and dissolved in phosphate-buffered saline just before use. Prior to their exposure, the cells were cultured in CHAT (10 μM deoxycytidine, 200 μM hypoxanthine, 0.1 μM aminopterin, 17.5 μM thymidine) medium for 2 days to reduce the background mutant fraction. Cultures of 20 ml at 5.0×10^5 cells/ml and of 50 ml at 2.0×10^5 cells/ml were treated at 37 °C with serial dilution of MCLR for 4 h and 24 h, respectively. They were then washed once, re-suspended in fresh medium, and cultured in new flasks for the MN assay and *TK* gene mutation assay, or diluted to be plated for survival estimates.

2.2. MN assay

Forty-eight hours after exposure, the MN assay samples were prepared as previously reported [20]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold fixative (glacial acetic acid: methanol, 1:3), and then re-suspended in methanol containing 1% acetic acid. A drop of the suspension was placed on a clean glass slide and air-dried. The cells were stained with 40 μg/ml acridine orange solution and immediately observed with the aid of an Olympus model BX50 fluorescence microscope equipped with a U-MWBV band pass filter. At least 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored.

2.3. TK gene mutation assay

The TK6 cell cultures were maintained for 3 days after exposure to permit expression of the *TK* deficient phenotype. To isolate the *TK* deficient mutants, we seeded cells into 96-well microwell plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluorothymidine (TFT). Cells from each culture were also plated at 1.6 cells/well in the absence of TFT for the determination of plating efficiency (PE). All plates were incubated for 14 days at 37 °C in a 5% CO_2, humidified incubator, and then scored for colony formation. Plates containing TFT were then re-fed with TFT, incubated for an additional 14 days, and scored for the appearance of slow-growing *TK* mutants. Mutation frequencies were calculated according to the Poisson distribution [21].

2.4. LOH analysis of TK mutants

Genomic DNA was extracted from *TK* mutant cells and used as a template for PCR. The PCR-based LOH analysis at human *TK* gene was described previously [19]. Two sets of primers were used to amplify the parts of exons 4 and 7 of the *TK* gene containing frameshift mutations. Another primer set for amplifying parts of the β-globin was also prepared. Quantitative-multiple PCR was subjected to co-amplification of the three regions and qualify and quantify the PCR products. They were analyzed with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and classified them into non-LOH, hemizygous LOH, or homozygous LOH mutants.

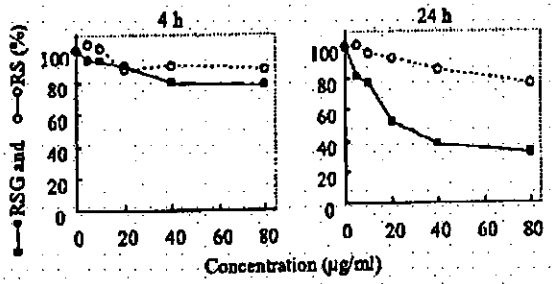

Fig. 1. Cytotoxic responses represented by RS and relative cell growth (RSG) of TK6 cells treated with MCLR for 4 or 24 h.

3. Results

3.1. Cytotoxic response to MCLR

TK6 cells were exposed to various doses of MCLR for 4 or 24 h. Fig. 1 shows cytotoxic responses; relative survival (RS) and relative suspension growth (RSG), which is relative cell growth during 72 h after exposure. Exposure to MCLR for 4 h did significantly affect RS or RSG. Exposure for 24 h, however, decreased RSG concentration-dependently, but did not significantly alter RS.

3.2. Genotoxic responses to MCLR

Exposure to MCLR for 24 h induced both MN and *TK* mutation in a concentration-dependent manner (Fig. 2). The maximum induction of MN and *TK* mutations were 4.8- and 5.1-times the control values. Two distinct phenotypic classes of *TK* mutants were generated. Normally growing (NG) mutants grew at the same rate as the wild type cells (doubling time 13–17 h), and slowly growing (SG) mutants grew at a slower rate (doubling time >21 h). NG mutants result mainly from intragenic mutations, while SG mutants result from gross genetic changes beyond the *TK* gene. The proportion of SG mutants increased in MCLR induced mutants, suggesting that MCLR was clastogenic.

3.3. Molecular analysis of TK mutants

Spontaneously arising and MCLR-induced *TK* mutants were isolated independently. The MCLR-induced

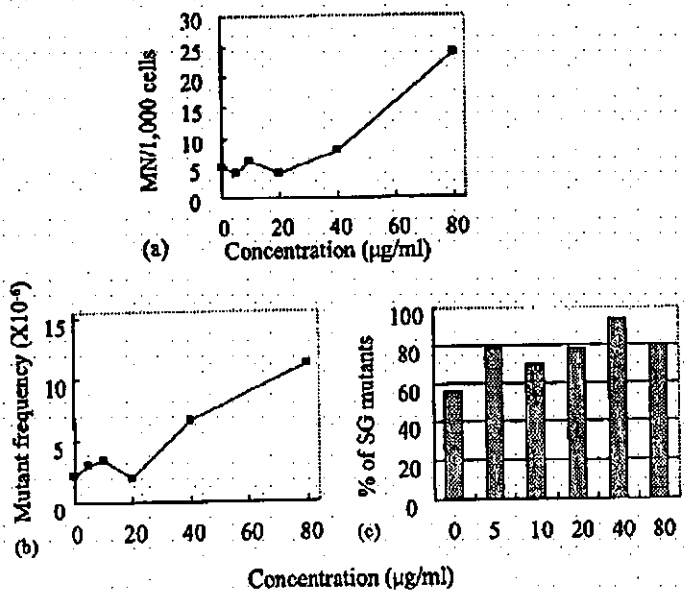

Fig. 2. MN induction (a), mutation frequency at *TK* locus (b), and percentage of slowly growing (SG) mutants (c) among TK-deficient mutants treated with MCLR for 24 h.

mice in vivo, it seems less toxic in vitro, particularly in non-liver cells. That may be because cyclic heptapeptide microcystines do not generally penetrate most cells including bacteria, and a specific transport system may be required [25]. We used 2 parameters to estimate MCLR cytotoxicty—RS and RSG. RS is relative plating efficiency just after exposure, while RSG is relative cell growth for the 3 days following exposure. RSG exhibited stronger response than RS, suggesting that MCLR has an inhibitory effect on cell growth [26]. Because the cytotoxicity was not severe, the genotoxic responses to MCLR must have been due to physiological effects. In the *TK* gene mutation assay, MCLR elevated not only the frequency of mutants, but also the fraction of SG mutants, suggesting that MCLR induced predominantly gross structural changes, such as large deletions, recombinations, and rearrangements.

Molecular analysis strongly supported this hypothesis. Most of the *TK* mutants induced by MCLR were the result of LOH, while the fraction of non-LOH mutants hardly changed (Fig. 3). LOH is an important genetic event in tumorigenesis and is frequently observed in a variety of human tumors. The two major mechanisms for generating LOH are deletion (hemizygous LOH) and inter-allelic recombination (homozygous LOH) [18,19]. Both mechanisms involve the repair of chromosomal double strand breaks (DSBs), either non-homologous end-joining and homologous recombination (HR), although their regulation and role have not been clarified [27]. Other mechanisms may be involved, too, including illegitimate recombination and mitotic non-disjunction [26]. DSB-inducing agents, such as ionizing irradiations, effectively produce LOH mutations through the repair pathways [17,18]. MCLR clastogenic activity may also involve DSBs. Honma and Little [28] demonstrated that 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), which is the most active tumor promoter known, preferably induces homozygous LOH through HR. MCLR also has tumor promoting activity; like the tumor promoter Okadaic acid, it inhibits protein phosphatase types 1 and 2A [29]. A cyanobacterial toxin, nodularin, which also inhibits protein phosphatases 1 and 2A with the same potency as does MCLR has been recognized as rat liver carcinogen rather than a tumor promoter [30]. The genotoxicity of nodularin, however, has not been clear. Matsushima et al. [31] demonstrated that MCLR promotes rat liver cancer initiated with diethyl-nitrosamine. The tumor promoting activity of MCE has been also shown in a two-stage transformation assay in vitro using Syrian hamster embryonic cells [32]. The induction of LOH by MCLR through recombination may be associated with its tumor promoting activity. It is reported that Okadaic acid induces minisatellite mutation in NIH3T3 cells probably through recombination events [33]. The potent hepatocarcinogen aflatoxin B1 also preferably induces LOH through HR in TK6 cells and mouse lymphoma L5178Y cells [34,35].

In conclusion, MCLR was clastogenic in human cells in the present study. It induced LOH, but not point mutations. The genotoxic activity may have been associated with the inductions of DSBs and/or its promoting activity. The association between a high incidence of primary liver cancer and drinking of pond and ditch water polluted by high level of cyanobacteria producing MCLR [3,36,37] suggests that liver is a target organ for MCLR carcinogenisity. Further studies using liver cells and tissues are required to clarify the mechanisms of MCLR genotoxicity in the liver.

Acknowledgements

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References

[1] W.W. Carmichael, Toxins of freshwater algae, In: A.T. Tu (Ed.), Handbook of natural Toxins, Marcel Dekker, New York, 1988, pp. 121–147.
[2] G.A. Codd, S.G. Bell, P. Brooks, Cyanobacterial toxins in water, Water Sci. Technol. 21 (1989) 1–13.
[3] W.W. Carmichael, The toxins of cyanobacteria, Sci. Am. 270 (1994) 78–86.
[4] F.D. Galey, V.R. Beasley, W.W. Carmichael, G. Kleppe, S. Hooser, W.M. Haschek, Blue-green algae (*Microcysis aeruginosa*) hepatotoxicosis in dairy cows, Am. J. Vet. Res. 48 (1987) 1415.
[5] I.R. Falconer, A.M. Bersfoed, M.T.C. Runnegar, Evidence of liver damage by toxin from a bloom of blue-green algae, *Microcystis aeruginosa*, Med. J. Aust. 1 (1983) 511–514.
[6] I.R. Falconer, Effects on human health of some toxic cyanobacteria (blue-green algae) in reservoirs, Toxicity Assess. 4 (1989) 175–184.
[7] W.P. Brooks, G.A. Codd, Distribution of *Microcystis aeruginosa* peptide toxin and interactions with hepatic microsomes in mice, Pharmacol. Toxicol. 60 (1987) 187–191.

Table 1
Cytotoxic and mutational response to MCLR[a] and LOH analysis of TK-mutants

Treatment	Cytotoxic and mutational response			LOH analysis at *TK* gene			
	RSG (%)	MF ($\times 10^{-6}$)	% SG	No. analyzed	None LOH	Hemizygous LOH	Homozygous LOH
Spontaneous	100	2.19	56	56			
NG mutants				19	14 (74)	3 (16)	2 (11)
SG mutants				37	0 (0)	9 (24)	28 (76)
MCLR-induced	32.6	11.2	80	36			
NG mutants				9	4 (44)	5 (56)	0 (0)
SG mutants				27	0 (0)	10 (37)	17 (63)

[a] 80 μg/ml for 24 h.

mutants were produced by the treatment at 80 μg/ml for 24 h. The cytotoxicity (RSG), mutation frequency, and proportion of SG mutants by the treatment are shown in Table 1. We used PCR-based LOH analysis of genomic DNA from *TK* mutants to classify the mutants into 3 types; Non-LOH, hemizygous LOH, and homozygous LOH. We analyzed 58 spontaneous and 36 MCLR induced *TK* mutants, including NG and SG type (Table 1). Every SG mutant was a result of LOH regardless of the treatment, suggesting that SG mutants were always associated with gross genetic changes. Among the MCLR-induced mutants, 56% of NG mutants and 100% of SG mutants exhibited LOH. Every LOH in the NG mutants was hemizygous, and 63% of LOH in the SG mutants was homozygous. This is in contrast to spontaneous *TK* mutants, where the majority of spontaneous NG and SG mutants were non-LOH (74%) and homozygous LOH (76%), respectively. Fig. 3 shows the spectra of spontaneous and MCLR-induced *TK* mutants in TK6 cells, which were adjusted by considering % SG mutants. These data clearly indicate that MCLR induced LOH, but not point mutation or other small genetic changes.

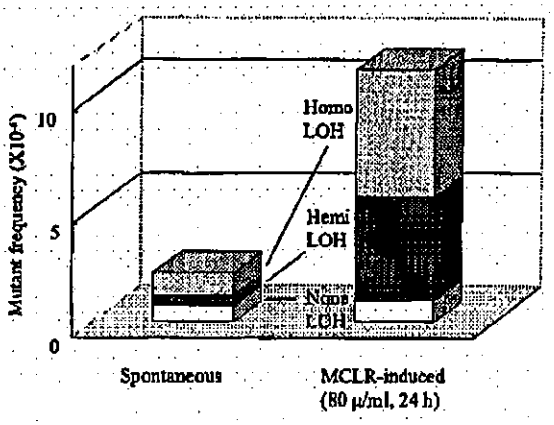

Fig. 3. Frequency and spectra of *TK* mutations in spontaneous and MCLR (80 μg/ml, 24 h) induced *TK* mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of NG and SG mutants and the result of molecular analysis (Table 1).

4. Discussion

Although MCLR causes severe hepatoxoxicity in mammals [9–12], its genotoxicity and carcinogenicity are inconclusive. Ding et al. [13] reported that microcystic cyanobacteria extract (MCE) significantly induced mutations in the Ames assay regardless of metabolic activation, although pure MCLR did not. Tsuji et al. [22,23] also failed to demonstrate MCLR genotoxicity in the Ames assay. On the other hand, MCLR has some genotoxic effects in mammalian cells. Ding et al [13] observed DNA damage in primary rat hepatocytes in comet assay, and Rao and Bhattacharya [14] found that MCLR could induce DNA fragmentation and strand breaks in mouse liver in vivo. Two studies reported the induction of chromosome aberrations and gene mutations in mammalian cells [15,24].

Our present study clearly demonstrated the in vitro genotoxicity of MCLR, which induced MN formation as well as gene mutations in human cells. A 24 h treatment was required, however, to express the effects. Although MCLR is toxic and highly lethal to

[8] A.S. Dahholker, W.W. Carmichael, Ultrastructural changes in the mouse liver induced by hepatotoxin from the freshwater cyanobacterium Microcystis aeruginosa strain 7820, Toxicon 25 (1987) 285–292.
[9] G.A. Codd, S.G. Bell, P. Brooks, Cyanobacterial toxins in water, Water Sci. Technol. 21 (1989) 1–13.
[10] S.B. Hooser, V.R. Beasley, R.A. Lovell, W.W. Carmichael, W.M. Haschek, Toxicity of microcystin-LR, a cyclic heptapeptide hepatotoxin from *Microcystis aeruginosa* to rats and mice, Vet. Pathol. 26 (1989) 246–252.
[11] P.V. Rao, R. Bhattacharya, S.C. Pant, A.S.B. Bhaskar, Toxicity evaluation of in vitro cultures of freshwater cyanobacterium *Microcystis aeruginosa*. Part 1. Hepatotoxic and histopathological effects in rats, Biomed. Environ. Sci. 8 (1995) 254–264.
[12] R. Bhattacharya, P.V.L. Rao, A.S.B. Bhaskar, S.C. Pant, S.N. Dube, Liver slice culture for assessing hepatotoxicity of freshwater cyanobacteria, Hum. Exp. Toxicol. 15 (1996) 105–110.
[13] W.X. Ding, H.M. Shen, H.G. Zhu, B.L. Lee, C.N. Ong, Genotoxicity of microcystic cyanobacteria extract of a water source in China, Mutation Res. 442 (1999) 69–77.
[14] P.V. Rao, R. Bhattacharya, The cyanobacteral toxin microcystin-LR induced DNA damage in mouse liver in vivo, Toxicology 114 (1996) 29–36.
[15] H. Suzuki, M.F. Watanabe, Y. Wu, T. Sugita, K. Kita, T. Sato, X.L. Wang, H. Tanzawa, S. Sekiya, N. Suzuki, Mutagenicity of microcystin-LR in human RSa cells, Int. J. Mol. Med. 2 (1998) 109–112.
[16] H.L. Liber, W.G. Thilly, Mutation assay at the thymidine kinase locus in diploid human lymphoblasts, Mutat. Res. 94 (1982) 467–485.
[17] C.Y. Li, D.W. Yandell, J.B. Little, Molecular mechanism of spontaneous and induced loss of heterozygosity in human cells in vitro, Somat. Cell Mol. Genet.18 (1992) 77–87.
[18] M. Honma, M. Hayashi, T. Sofuni, Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells, Mutat. Res. 374 (1997) 89–98.
[19] M. Honma, M. Momose, H. Tanabe, H. Sakamoto, Y. Yu, J.B. Little, T. Sofuni, M. Hayashi, Requirement of wild-type p53 protein for maintenance of chromosomal integrity, Mol. Carcinogenesis 28 (2002) 203–214.
[20] L.S. Zhang, M. Honma, A. Matsuoka, T. Suzuki, T. Sofuni, M. Hayashi, Chromosome painting analysis of spontaneous and methylmethanesulfonate, induced trifluorothymidine-resistant L5178Y cell colonies, Mutat. Res. 370 (1996) 181–190.
[21] E.E. Furth, W.G. Thilly, B.W. Penman, H.L. Liber, W.M. Rand, Quantitative assay for mutation in diploid human lymphoblasts using microtiter plates, Anal. Biochem. 110 (1981) 1–8.
[22] K. Tsuji, T. Watanuki, F. Kondo, M.F. Watanabe, S. Suzuki, H. Nakazawa, H. Uchida, K.I. Harada, Stability of microcystins from cyanobacteria—II. Effect of UV light on decomposition and isomerization, Toxicon 33 (1995) 1619–1631.
[23] K. Tsuji, T. Watanuki, F. Kondo, M.F. Watanabe, H. Nakazawa, S. Suzuki, H. Uchida, K.I. Harada, Stability of microcystins from cyanobacteria. Part IV. Effect of chlorination on decomposition, Toxicon 35 (1997) 1033–1041.
[24] W.M. Repavich, W.C. Sonzongni, J.H. Standridge, R.E. Wedepohl, L.F. Meisner, Cyanobacteria (blue-green algae) in Wisconsin waters: acute and chronic toxicity, Water Res. 24 (1990) 225–231.
[25] M. Runnegar, N. Berndt, N. Kaplowitz, Microcystin uptake and inhibition of protein phosphatases: effects of chemoprotectants and self-inhibition in relation to known hepatic transporters, Toxicol. Appl. Pharmacol. 134 (1995) 264–272.
[26] M. Honma, M. Momose, H. Sakamoto, T. Sofuni, M. Hayashi, Spindle poisons induce allelic loss in mouse lymphoma cells through mitotic non-disjunction, Mutat. Res. 493 (2001) 110–114.
[27] J.M. Stark, M. Jasin, Extensive loss of heterozygosity is suppressed during homologous repair of chromosomal breaks, Mol. Cell. Biol. 23 (2003) 733–743.
[28] M. Honma, J.B. Little, Recombinagenic activity of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate in human lymphoblastoid cells, Carcinogenesis 16 (1995) 1717–1722.
[29] S. Yoshizawa, R. Matsushima, M.F. Watanabe, K.I. Harada, W.W. Carmichael, H. Fujiki, Inhibition of protein phosphatases by microcystins and nodularin associated with hepatotoxicity, J. Cancer Res. Clin. Oncol. 116 (1990) 609–614.
[30] T. Ohta, E. Sucoka, N. Iida, A. Komori, M. Suganuma, R. Nishiwaki, M. Tatematsu, S.J. Kim, W.W. Carmichael, H. Fujiki, Nodularin, a potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver, Cancer Res. 54 (1994) 6402–6406.
[31] R. Matsushima, T. Ohta, S. Nishiwaki, M. Suganuma, K. Koyama, T. Ishikawa, W.W. Carmichael, H. Fujiki, Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR, J. Cancer Res. Clin. Oncol. 118 (1992) 420–424.
[32] H.B. Wang, H.G. Zhu, Promoting activity of microcystins extract from waterblooms in SHE cell transformation assay, Biomed. Environ. Sci. 9 (1996) 46–51.
[33] H. Nakagama, S. Kaneko, H. Shima, H. Inamori, H. Fukuda, R. Kominami, T. Sugimura, M. Nagao, Induction of minisatellite mutation in NIH 3T3 cells by treatment with the tumor promoter okadaic acid, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 10813–10816.
[34] P.M. Stettler, C. Sengstag, Liver carcinogen aflatoxin B1 as an inducer of mitotic recombination in a human cell line, Mol. Carcinogen 31 (2001) 125–138.
[35] V. Preisler, W.J. Caspary, F. Hoppe, R. Hagen, H. Stopper, Aflatoxin B1-induced mitotic recombination in L5178Y mouse lymphoma cells, Mutagenesis 15 (2000) 91–97.
[36] S.Z. Yu, G. Chen, Blue-green algae toxins and liver cancer, Chin. J. Cancer Res. 6 (1994) 9–17.
[37] Y. Ueno, S. Nagata, T. Tsutsumi, A. Hasegawa, M.F. Watanabe, H.D. Park, G.C. Chen, G. Chen, S.Z. Yu, Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay, Carcinogenesis 17 (1996) 1317–1321.

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Cytogenetic and Genome Research

A liver micronucleus assay using young rats exposed to diethylnitrosamine: methodological establishment and evaluation

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Abstract. We have developed a simple liver micronucleus assay using young rats (up to 4 weeks old) to assess cytogenetic damage of chemicals in liver cells. Diethylnitrosamine (DEN) was used as a model rodent hepatocarcinogen in this study. Compared to the partial hepatectomy method most commonly used for the liver micronucleus assay, the present assay method showed equal or even higher practicability. The young rat liver micronucleus assay was also characterized for rat strain differences, sampling time after treatment, single treatment vs. split treatment, age of animals, administration route, and staining method. Although based on one model chemical, we propose an acceptable protocol for the micronucleus assay using young rat liver as follows: Up to 4-week-old rats should be used; oral or intraperitoneal administration can be used; single or repeated treatment protocols can be applied; sampling time is 3–5 days after the last treatment; hepatocytes are prepared by the collagenase perfusion method; and cells are stained with the AO-DAPI double staining method.

Micronucleus assays have been widely performed using bone marrow cells to assess the clastogenic potential of chemicals in vivo. Since bone marrow (BM) is one of the continuously proliferating tissues in adult animals, it has been used as a common target organ for cytogenetic studies. However, it is well known that some compounds need metabolic activation in the liver, and it has been pointed out that some pro-mutagens elicit a negative response in the BM micronucleus assay (Morita et al., 1997). It may be considered that some active metabolites have a very short lifespan and do not reach the BM at sufficient concentrations to induce micronuclei. In fact, some rodent liver carcinogens, including di-alkyl-nitrosamines, nitro aromatic compounds, and azo derivatives, gave negative results in a BM assay (Angelosanto, 1995). It is worthwhile, therefore, to consider the selection of other organs for evaluating genotoxicity of test chemicals, especially the liver for detecting liver carcinogens. The use of suitable organs for genotoxicity determination is also recommended in the guidance proposed by the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (Committee on the Mutagenicity of Chemicals, 2000).

In recent years, various investigators have tried to develop a liver micronucleus assay. Due to extremely low mitotic activity in adult animals, procedures require mitotic stimulation of liver cells together with treatment of animals with test chemicals. Tates et al. (1980) reported a method comprising partial hepatectomy (PH) before or after chemical treatment for the liver micronucleus assay. The division of hepatocytes is stimulated by PH, and positive responses were obtained in induction of micronuclei in liver cells after treatment with liver carcinogens that gave negative results in the BM assay. Other investigators also evaluated the chemical clastogenicity potential in the liver by the PH method (Cliet et al., 1989; Roy and Das, 1990; Mere-

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to et al., 1994; Zhurkov et al., 1996). There are, however, two shortcomings in the PH method: 1) Technically it is not easy to perform successful hepatectomy on all animals used in the assay, and 2) it has been reported that cytochrome P450, styrene mono-oxygenase, epoxide hydrolase, and glutathione-S-epoxide transferase activities decreased by 50, 35, 50, and 35%, respectively 12 h after PH (Rossi et al., 1987).

Another method used 4-acetyl aminofluorene (4AAF), a mitogen for liver cells (Braithwaite and Ashby, 1988), to activate cell proliferation. In this method, the possibility of interaction with the test chemical has not been ignored (Parton and Garriott, 1997). An in vivo/in vitro assay system has also been reported, i.e., after treatment of animals with test chemicals in vivo, hepatocytes were collected and primary cell cultures were established with growth factors before cell harvest for slide preparation (Sawada et al., 1991). However, this in vivo/in vitro method is labor-intensive, costly and time consuming; thus it has not been used as a routine method to evaluate chemical clastogenicity in the liver.

The use of proliferating tissue is a prerequisite for the micronucleus assay. In 4-week-old rats, the hepatocytes are still proliferating, and the percentage of S phase cells is more than 40 times higher than in adult rat liver (Parton and Garriott, 1997). Sipes and Gandolfi (1993) reported that P450 levels reach a maximum at 4 weeks of age in the rat. Considering these points, we developed and evaluated the young (4-week-old) rat liver micronucleus assay. We used diethylnitrosamine (DEN), which is a well-known rodent liver carcinogen, as a model compound. DEN was negative in the conventional BM micronucleus assay (Morita et al., 1997), but positive in the liver micronucleus assay using the PH method (Tates et al., 1980). This paper describes the comparison of the PH and young rat methods and investigations of technical points in the young rat liver micronucleus assay.

Materials and methods

Animals

Male Fischer 344 (F344) and Sprague Dawley (SD) rats were purchased from Charles River Japan. The animals were housed under a 12-hour light-dark cycle and allowed free access to food and water.

Chemicals

Diethylnitrosamine (DEN, CAS No. 55-18-5), was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DEN was dissolved in physiological saline immediately before treatment and given once or twice by intraperitoneal injection or gavage to rats.

Comparison of the young rat method with the partial hepatectomy (PH) method

F344 or SD rats were treated orally with 50 mg/kg of DEN at 3 or 4 weeks of age in the young rat method, and at 6 weeks of age in the PH method. In the former method, slide preparations were made 2–5 days after single treatment, and peripheral blood samples were collected and supravitally stained with acridine orange (AO)-coated slides (Hayashi et al., 1990) before and 1, 2, and 3 days after treatment. In the latter method, PH was performed 7 days after treatment, and the slides were prepared 2–5 days after PH.

Dose dependency and age effect

DEN at dose levels of 12.5, 25, and 50 mg/kg was administered to 4-week-old rats, and hepatocytes were isolated 5 days after treatment. To investigate the effect of age, F344 rats at 3–9 weeks of age were treated with DEN (50 mg/kg) once and slide preparations were made on the 5th day after treatment as with the dose-response assay.

Administration route difference and treatment times

4-week-old F344 rats were treated by gavage or intraperitoneally with DEN once at 40 mg/kg or twice at 20 mg/kg with a 24-hour interval. Hepatocytes were isolated 2–5 days after the last treatment.

Preparation of hepatocytes

Hepatocytes were isolated from anesthetized rats by the collagenase perfusion method, rinsed with 10% neutral buffered formalin 2 or 3 times and centrifuged at 50 *g* (500 rpm) for 1 min (Clict et al., 1989). Hepatocytes were suspended with 10% neutral buffered formalin and stored in a refrigerator until analysis. In the case of PH, two-thirds of the liver was removed according to the published method (Higgins and Anderson, 1931). The hepatocytes were suspended with 10% neutrd buffered formalin, and kept in a refrigerator unill analysis.

Microscopy and micronucleus determination

Immediately prior to analysis, 10–20 µl of hepatocyte suspensions were mixed with an equal volume of AO-4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain solution for fluorescent microscopy. The AO-DAPI stain solution contains one part of 500 µg/ml of AO aqueous solution and one part of 10 µg/ml of DAPI aqueous solution. Approximately 10–20 µl of stained hepatocyte suspension was dropped onto a glass slide and covered with a coverslip (24 × 40 mm).

Hepatocytes were analyzed under a fluorescent microscope (×400 or higher) equipped with a UV excitation system. The number of micronucleated hepatocytes (MNHEPs) was recorded based on analysis of 2000 hepatocytes (in two fields) from each animal. In accordance with the methods of Braithwaite and Ashby (1988) and Clict et al. (1989), the following classification criteria for MNHEPs were used: Round or distinct micronuclei stained with the same color as the nuclei, with diameters of 1/4 or less that of the main nuclei. The number of mitotic cells was also counted in 1000 hepatocytes in each animal to determine the mitotic index (MI) for administration route or treatment time differences. Mitotic cells were defined as cells at any stage from prophase to telophase.

Peripheral blood micronucleus assay

5–10 µl of peripheral blood samples were collected from the tail vein and dropped on AO-coated slides (Hayashi et al., 1990), covered with a coverslip and stored overnight in a refrigerator until analysis. Reticulocytes supravitally stained with AO were analyzed under a fluorescent microscope (×600 or higher) equipped with a blue light excitation system. The number of micronucleated reticulocytes (MNRETs) was recorded by evaluation of 2000 reticulocytes in two fields per animal.

Statistical analysis

The incidences of micronucleated hepatocytes or reticulocytes were analyzed statistically by using Kastenbaum's and Bowman's tables (Kastenbaum and Bowman, 1970).

Results

AO-DAPI double fluorescent staining

Figure 1 shows fluorescent microphotographs of nuclei in young rat hepatocytes stained with AO alone (Fig. 1a), DAPI alone (Fig. 1c), and combination of AO and DAPI (Fig. 1b). Compared to AO or DAPI alone, the double staining allows better distinction of micronuclei and cytoplasm.

Comparison of the young rat method with the partial hepatectomy method

The results of the assay with DEN at 50 mg/kg are shown in Fig. 2. Both methods revealed significant increases in the numbers of MNHEPs in comparison with concurrent controls. The frequencies of MNHEPs in young rats were generally higher

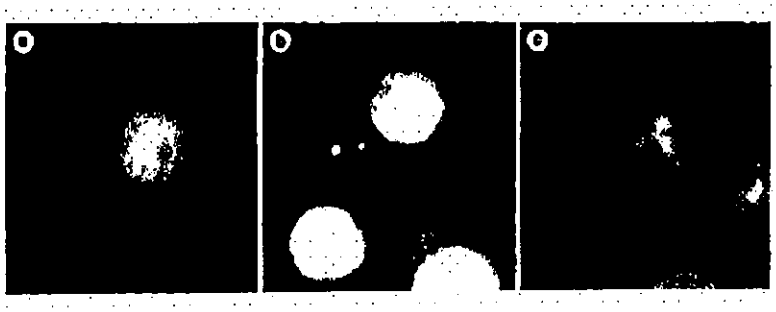

Fig. 1. Fluorescent microphotographs of hepatocyte nuclei from young rats stained with (a) AO alone, (b) a combination of AO and DAPI, and (c) DAPI alone.

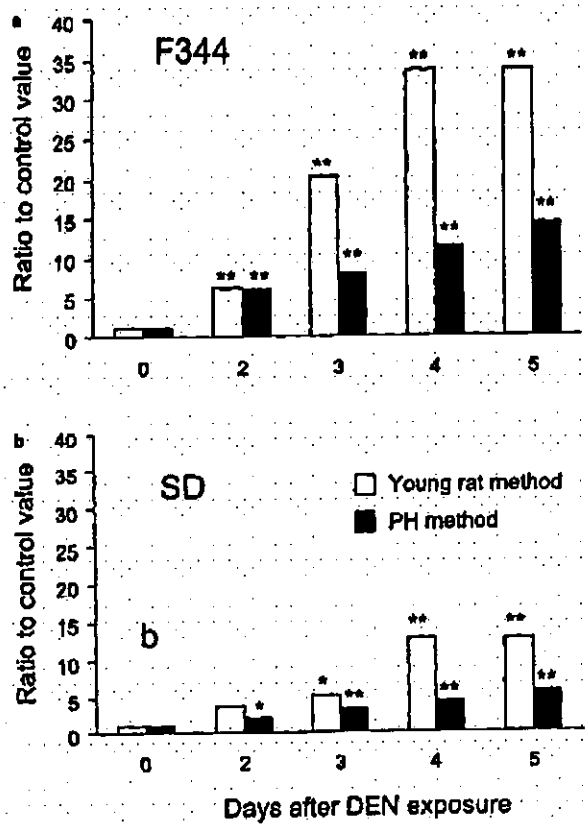

Fig. 2. Frequencies of MNHEPs (micronuclei/1000 hepatocytes) 2 to 5 days after administration of 50 mg/kg DEN to (a) F344 rats and (b) SD rats in the young rat method and PH method. Mean of 4–5 animals. * $P < 0.05$, ** $P < 0.01$, significantly different from the concurrent solvent control.

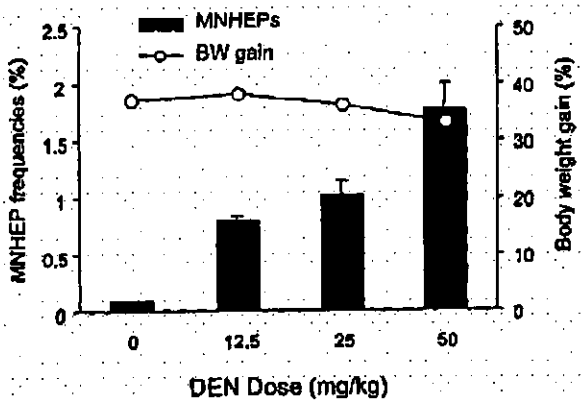

Fig. 3. MNHEP frequencies (evaluation of 2000 hepatocytes) 5 days after administration of DEN (12.5, 25 and 50 mg/kg) to F344 rats (column with standard deviation bar). Body weight (BW) gain was only marginally affected at the highest dose.

than those of the PH method and it was shown that the MNHEP frequencies of F344 rats (Fig. 2a) were higher than those of SD rats (Fig. 2b) in both methods. There was no statistically significant difference in MNRET induction observed in peripheral blood at any treatment time in either F344 or SD rats (data not shown).

Dose dependency and age effect

The MNHEP frequencies after administration of DEN (12.5, 25, 50 mg/kg) to 4-week-old F344 male rats increased dose dependently and reached approximately 9- to 20-fold the concurrent solvent control (Fig. 3). During this study, the rate of body weight gain was only marginally decreased at the highest DEN dose. The MNHEP frequency after 50 mg/kg DEN administration to F344 rats aged between 3 and 9 weeks decreased age-dependently, with the highest value (2.3%) at 3 weeks of age, and lowest (0.17%) at 9 weeks of age (Fig. 4).

Route difference and treatment times

To determine the influence of administration route, DEN was given either orally or intraperitoneally to 4-week-old F344 rats. MNHEP frequency was similar for both routes, 2–5 days after single (40 mg/kg) or the second treatment for a split-dose (2 × 20 mg/kg) regime (Fig. 5a, b). The MNHEP frequency increased depending on the time after DEN treatment (up to 5 days). On days 2, 3, and 4, split DEN dosing resulted in higher MNHEP frequencies compared to the single dose protocol. Mitotic cells were observed in 1.2 ± 0.30% to 1.5 ± 0.74% of cells in the solvent control group 5 days after treatment, and in

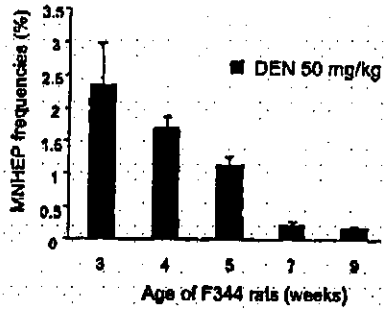

Fig. 4. MNHEP frequencies (evaluation of 2000 hepatocytes) after administration of 50 mg/kg DEN to F344 rats aged 3 to 9 weeks. Data are the mean of five animals with standard deviation.

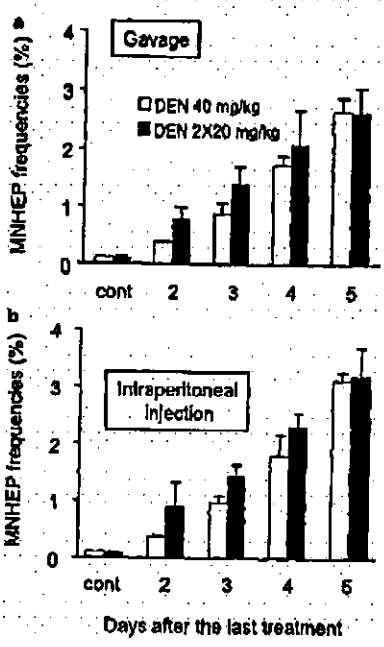

Fig. 5. DEN-induced MNHEP frequency (evaluation of 2000 hepatocytes) 2 to 5 days after a single 40 mg/kg dose or two 20 mg/kg doses 24 h apart in 4-week-old F344 rats by (a) gavage or (b) intraperitoneal injection. Results are the mean of five animals with standard deviation.

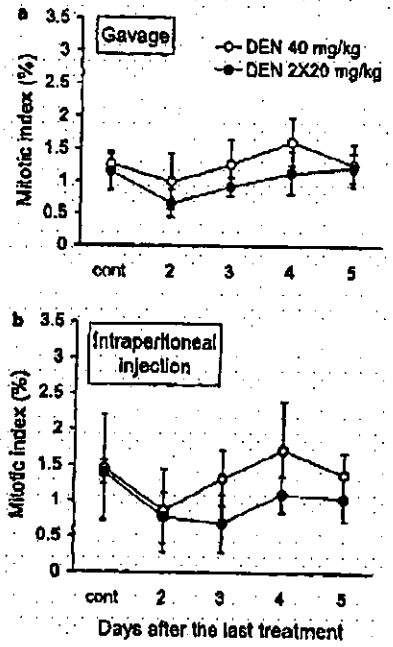

Fig. 6. Frequencies of mitotic indexes (evaluation of 1000 hepatocytes) 2 to 5 days after a single 40 mg/kg dose or two 20 mg/kg doses 24 h apart in 4-week-old F344 rats by (a) gavage or (b) intraperitoneal injection. Results are the mean of five animals with standard deviation.

0.7 ± 0.21 % to 1.7 ± 0.68 % of cells in the DEN group (Fig. 6a, b). The incidence of metaphase cells tended to be lower after the double treatment of DEN.

Discussion

Since Tates et al. reported a liver micronucleus assay in 1980, a number of variations have been published (Cliet et al., 1989; Roy and Das, 1990; Mereto et al., 1994; Zhurkov et al., 1996). In these studies, the PH method, the chemical mitogens method, and an in vivo/in vitro method have been applied to assess micronucleus induction in liver cells. These methods have been used on a case by case basis, but they have not been used routinely for evaluation of genotoxicity of chemicals. Some criticisms of these methods are 1) introduction of abnormal physiological conditions (e.g., PH method), 2) increased unknown factors as a result of interaction between test chemical and mitogen, 3) laborious and time-consuming (e.g., in vivo/in vitro method). We have thus paid attention to the method based on proliferating activity of liver cells in young rats up to about 4 weeks of age. Although the method using young rat liver was reported by Parton and Garriott (1997), it has not been well characterized and evaluated.

We consider the young rat liver micronucleus assay method as advantageous because it does not require any physical injury such as PH, nor pretreatment with mitogens that may interact with a test substance, nor setting up of primary culture that also may cause damage to the target cells. Therefore, we propose to use the present method as a tool for evaluation of chemical genotoxicity that may occur in the liver.

As shown in Fig. 6, we confirmed that there were many mitotic cells in the liver of 4-week-old rats. The mitotic index (MI) observed in the present study was comparable to that reported by Parton and Garriott (1997). As shown in Fig. 4, the incidence of MNHEP decreased with the age of the rats. This can be explained by an age-related decrease in proliferation of hepatocytes and dilution of the cells with micronuclei with undivided normal hepatocytes. This phenomenon was also observed in the PH method. The MI in regenerating hepatocytes is 3.6% and decreases below 1% at 72 h after PH (Grisham, 1962; Parton and Garriott, 1997). To restrict the cell population to one cell division after treatment, co-treatment with cytochalasin B could be considered. It would, however, be important to consider possible interactions between test chemical and cytochalasin B. Therefore we believe that it is acceptable to perform the assay without co-treatment of cytochalasin B as long as rats at 4 weeks of age are used.

The AO-DAPI staining method, which was originally developed for the testis micronucleus assay (Noguchi, 1997), was efficient for analysis of micronuclei in cytoplasm of hepatocytes. This novel staining method gave clearer distinction of