strand break targeted to a mammalian chromosome. EMBO J 17: 325–333.

Quintana PJ, Neuwirth EA, Grosovsky AJ. 2001. Interchromosomal gene conversion at an endogenous human cell locus. Genetics 158:757–767.

Richardson C, Jasin M. 2000. Frequent chromosomal translocations induced by DNA double-strand breaks. Nature 405:697–700.

Rothkamm K, Kruger I, Thompson LH, Lobrich M. 2003. Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol 23:5706–5715.

Saintigny Y, Lopez BS. 2002. Homologous recombination induced by replication inhibition, is stimulated by expression of mutant p53. Oncogene 21:488–492.

Sands AT, Suraokar MB, Sanchez A, Marth JE, Donehower LA, Bradley A. 1995. p53 deficiency does not affect the accumulation of point mutations in a transgene target. Proc Natl Acad Sci USA 92: 8517–8521.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63:1129–1136.

Selivanova G, Wiman KG. 1995. p53: a cell cycle regulator activated by DNA damage. Adv Cancer Res 66:143–180.

Shao C, Deng L, Henegariu O, Liang L, Stambrook PJ, Tischfield JA. 2000. Chromosome instability contributes to loss of heterozygosity in mice lacking p53. Proc Natl Acad Sci USA 97:7405–7410.

Skopek TR, Liber HL, Penman BW, Thilly WG. 1978. Isolation of a human lymphoblastoid line heterozygous at the thymidine kinase locus: possibility for a rapid human cell mutation assay. Biochem Biophys Res Commun 84:411–416.

Stambrook PJ, Shao C, Stockelman M, Boivin G, Engle SJ, Tischfield JA. 1996. APRT: a versatile in vivo resident reporter of local mutation and loss of heterozygosity. Environ Mol Mutagen 28:471–482.

Stark JM, Jasin M. 2003. Extensive loss of heterozygosity is suppressed during homologous repair of chromosomal breaks. Mol Cell Biol 23:733–743.

Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, Takei Y, Nakamura Y. 2000. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature 404:42–49.

Tarapore P, Fukasawa K. 2000. p53 mutation and mitotic infidelity. Cancer Invest 18:148–155.

Tlsty TD. 2002. Functions of p53 suppress critical consequences of damage and repair in the initiation of cancer. Cancer Cell 2:2–4.

Turker MS. 2003. Autosomal mutation in somatic cells of the mouse. Mutagenesis 18:1–6.

Umeki S, Suzuki T, Kusunoki Y, Seyama T, Fujita S, Kyoizumi S. 1997. Development of a mouse model for studying in vivo T-cell receptor mutations. Mutat Res 393:37–46.

van Den BM, Lohman PH, Pastink A. 2002. DNA double-strand break repair by homologous recombination. Biol Chem 383:873–892.

van Kreijl CF, McAnulty PA, Beems RB, Vynckier A, van Steeg H, Fransson-Steen R, Alden CL, Forster R, van der Laan JW, Vandenberghe J. 2001. Xpa and Xpa/p53$^{+/-}$ knockout mice: overview of available data. Toxicol Pathol 29(Suppl):117–127.

van Steeg H, Mullenders LH, Vijg J. 2000. Mutagenesis and carcinogenesis in nucleotide excision repair-deficient XPA knock out mice. Mutat Res 450:167–180.

van Steeg H. 2001. The role of nucleotide excision repair and loss of p53 in mutagenesis and carcinogenesis. Toxicol Lett 120:209–219.

Weinert T, Lydall D. 1993. Cell cycle checkpoints, genetic instability and cancer. Semin Cancer Biol 4:129–140.

Wiesmuller L. 2001. Genetic stabilization by p53 involves growth regulatory and repair pathways. J Biomed Biotechnol 1:7–10.

Wijnhoven SW, Kool HJ, van Teijlingen CM, van Zeeland AA, Vrieling H. 2001. Loss of heterozygosity in somatic cells of the mouse: an important step in cancer initiation? Mutat Res 473:23–36.

Xia F, Amundson SA, Nickoloff JA, Liber HL. 1994. Different capacities for recombination in closely related human lymphoblastoid cell lines with different mutational responses to X-irradiation. Mol Cell Biol 14:5850–5857.

Xia F, Wang X, Wang YH, Tsang NM, Yandell DW, Kelsey KT, Liber HL. 1995. Altered p53 status correlates with differences in sensitivity to radiation-induced mutation and apoptosis in two closely related human lymphoblast lines. Cancer Res 55:12–15.

Xia F, Liber HL. 1997. The tumor suppressor p53 modifies mutational processes in a human lymphoblastoid cell line. Mutat Res 373:87–97.

Yu Y, Li CY, Little JB. 1997. Abrogation of p53 function by HPV16 E6 gene delays apoptosis and enhances mutagenesis but does not alter radiosensitivity in TK6 human lymphoblast cells. Oncogene 14:1661–1667.

Zhang H, Xiong Y, Beach D. 1993. Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol Biol Cell 4:897–906.

Zhen W, Denault CM, Loviscek K, Walter S, Geng L, Vaughan AT. 1995. The relative radiosensitivity of TK6 and WI-L2-NS lymphoblastoid cells derived from a common source is primarily determined by their p53 mutational status. Mutat Res 346:85–92.

Zurer I, Hofseth LJ, Cohen Y, Xu-Welliver M, Hussain SP, Harris CC, Rotter V. 2004. The role of p53 in base excision repair following genotoxic stress. Carcinogenesis 25:11–19.

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Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells

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Abstract

The recent finding that acrylamide (AA), a potent carcinogen, is formed in foods during cooking raises human health concerns. In the present study, we investigated the genotoxicity of AA and its metabolite glycidamide (GA) in human lymphoblastoid TK6 cells examining three endpoints: DNA damage (comet assay), clastogenesis (micronucleus test) and gene mutation (thymidine kinase (*TK*) assay). In a 4 h treatment without metabolic activation, AA was mildly genotoxic in the micronucleus and *TK* assays at high concentrations (>10 mM), whereas GA was significantly and concentration-dependently genotoxic at all endpoints at ≥0.5 mM. Molecular analysis of the *TK* mutants revealed that AA predominantly induced loss of heterozygosity (LOH) mutation like spontaneous one while GA-induced primarily point mutations. These results indicate that the genotoxic characteristics of AA and GA were distinctly different: AA was clastogenic and GA was mutagenic. The cytotoxicity and genotoxicity of AA were not enhanced by metabolic activation (rat liver S9), implying that the rat liver S9 did not activate AA. We discuss the in vitro and in vivo genotoxicity of AA and GA.

Keywords: Acrylamide; Gycidamide; Genotoxicity; *TK* mutation; Metabolic activation

1. Introduction

Acrylamide (AA) is a synthetic chemical that has been produced since the early 1950s. Because AA polymerizes easily to an adhesive gel, it has been widely used in industry for water flocculation, soil coagulation and grouts. Because it had been believed that humans are rarely exposed to AA under ordinary circumstances, concern was centered only on occupational exposure [1]. In 2000, however, Tareke et al. [2] reported that AA was unexpectedly discovered in cooking foods. It forms during frying and baking principally by a Maillard reaction between asparagine residues and glucose [3,4]. This finding raises concerns about the health risks of AA for the general population [5].

According to toxicological studies, AA is neurotoxic for animals and human [6,7], and the International

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Agency for Research on Cancer classifies it as 2A, a probable human carcinogen [1]. AA is also genotoxic in somatic and germinal cells in in vitro and in vivo [8]. In vivo examination [8] AA is metabolized to the epoxide derivative glycidamide (GA), presumably by cytochrome P4502E1 (CYP2E1) [9]. GA may be more toxic than AA because it reacts quickly with DNA and other biological macromolecules, and it is positive in most genotoxicity tests [8]. AA, on the other hand, is inactive in bacterial and some in vitro mammalian gene mutation assays, but it induces sister chromatid exchanges and chromosome aberrations in vitro and in vivo [8]. AA may have indirect genotoxic mechanisms, such as protein binding, spindle disturbance or hormonal imbalance, which could lead to tumors [10,11]. Thus, the genotoxic mechanism of AA is unclear.

In the present study, we used human lymphoblatoid TK6 cells to investigate the genotoxicity of AA and GA and its mechanisms. TK6 cells are widely used for the thymidine kinase (*TK*) gene mutation assay and can also be used in the in vitro micronucleus (MN) and comet (COM) assays. The *TK* gene mutation assay detects a wide range of genetic damage, including gene mutations, large-scale chromosomal deletions, recombination and aneuploidy [12], while other mammalian gene mutation assays, such as the *HPRT* and transgenic *LacZ* and *LacI* gene assays, detect only point mutations and small deletions [13]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumably relevant to carcinogenesis. Molecular analysis of the TK mutants induced by AA or GA can help elucidate their genotoxic mechanisms. In addition, because it uses a human cell line, the *TK* assay is appropriate for human hazard evaluation.

2. Materials and methods

2.1. Cell culture, chemicals and treatment

The TK6 human lymphoblastoid cell line has been described previously [14]. The 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 U/ml penicillin and 100 μg/ml streptomycin and maintained at 10^5 to 10^6 cells/ml at 37 °C in a 5% CO_2 atmosphere with 100% humidity.

AA (CAS # 79-06-1) and GA (CAS # 5694-00-8) were purchased from Wako Pure Chemical Co. (Tokyo). We dissolved them in phosphate-buffered saline just before use. *N*-di-*N*-butylnitrosamine (DBN) (CAS # 924-16-3) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and dissolved in DMSO for use. Post-mitochondrial supernatant fractions of liver homogenate (S9) were purchased from Kikkoman Co. Ltd. (Noda, Chiba, Japan), which were prepared from the liver of phenobarbital- and 5,6-benzoflavone-treated SD rats. We prepared a 10 ml S9 mix with 4 ml S9 fraction and 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP and 150 mM KCl.

We treated 20 ml aliquots of cell suspensions (5.0×10^5 cells/ml) at 37 °C for 4 h with serially diluted AA or GA, washed them once, re-suspended them in fresh medium, and cultured them in new flasks for the MN and *TK* assays or diluted and plated them for survival measurement (PE0). We treated the cultures with AA both in the absence and presence of 5% S9 mix.

2.2. Comet assay

After treating the cells for 4 h with AA or GA, we prepared slides for alkaline COM assay as previously reported [15]. Briefly, the cells were suspended in 0.5% agarose-LGT (Nakalai Tesque Inc., Kyoto, Japan), quickly layered on a slide (Matsunami Glass Ind. Ltd., Osaka, Japan) coated with 1% agarose GP-42 (Nakalai Tesque Inc.), and covered with 0.5% agarose-LGT. We immersed the slide in alkaline lysing solution (pH 13) for 1 h, electrophoresed it for 15 min after the unwinding treatment, fixed the cells with 70% ethanol, and stained them with SYBER green (Molecular Probes, Eugene, OR) according to the manufacturer's recommendation. We observed the cells by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and the tail length of the comet image was measured. We statistically analyzed the difference between the non-treated and treated plates with the Dunnett's test after one-way ANOVA [16].

2.3. Micronuclei test

Forty-eight hours after treatment, we prepared the MN test samples as previously reported [17]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold glacial acetic acid in methanol (1:3), and resuspended in methanol containing 1% acetic acid. We placed a drop of the suspension on a clean glass slide and allowed it to air-dry. We stained the cells with 40 μg/ml acridine orange solution and immediately observed them by Olympus model BX50 fluorescence microscope. At least, 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran–Armitage trend test [18].

2.4. TK gene mutation assay

The TK6 cell cultures were maintained for 3 days after treatment to permit expression of the *TK* deficient phenotype. To isolate the *TK* deficient mutants, we seeded cells from each

culture into 96-microwell plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluorothymidine (TFT). We also plated them at 1.6 cells/well in the absence of TFT for the determination of plating efficiency (PE3). All plates were incubated at 37 °C in 5% CO_2 in a humidified incubator. The TK assay produces two distinct phenotypic classes of *TK* mutants: normally growing (NG) mutants had the same doubling time (13–17 h) as the wild type cells, and slowly growing (SG) mutants had a doubling time of >21 h. The difference is thought to be due to a putative gene near the *TK* gene. NG mutants result mainly from intragenic mutations, such as point mutations and small deletions, while SG mutants result from gross genetic changes extending beyond the *TK* gene [19]. We scored for the colonies in the PE plates and for the colonies for normal-growing *TK* mutants in the TFT plates at 14th day after plating. We then refed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing *TK* mutants. Mutation frequencies were calculated according to the Poisson distribution [20]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [21].

2.5. Molecular analysis of TK mutants

Genomic DNA was extracted from *TK* mutant cells and used as a template for the polymerase chain reaction (PCR). We analyzed for loss of heterozygosity (LOH) at the human *TK* gene by PCR products as described previously [22]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that contains frameshift mutations. Another primer set for amplifying parts of the β-globin were also prepared. We used quantitative-multiple PCR to co-amplify the three regions and to identify and quantify the PCR products. We analyzed them with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and classified the mutants into "none LOH", "hemizygous LOH" or "homozygous LOH". To determine the extent of LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis described previously [22]. The results were processed by GenoTyper™ software (PE Biosystems) according to the manufacturer's guidelines.

3. Results

3.1. Cytotoxic and genotoxic responses to AA and GA

Fig. 1a shows the effect of AA on relative survival (RS), mutation frequency (*TK* assay) and number of micronucleated cells per 1000 cells examined. AA was concentration-dependently cytotoxic, permitting about 20% RS at the maximum concentration (14 mM), while its genotoxicity and clastogenicity were weak. We repeated the experiment because of the weak genotoxicity. AA showed negative in the first *TK* assay, but positive in the second statistically. In MN test, both experiments showed statistically positive. GA, in contrast, was significantly genotoxic even at concentrations that were not severely cytotoxic (Fig. 1b). At the maximum concentration (2.4 mM), GA induced *TK* mutation frequencies that were about 20 times and MN fre-

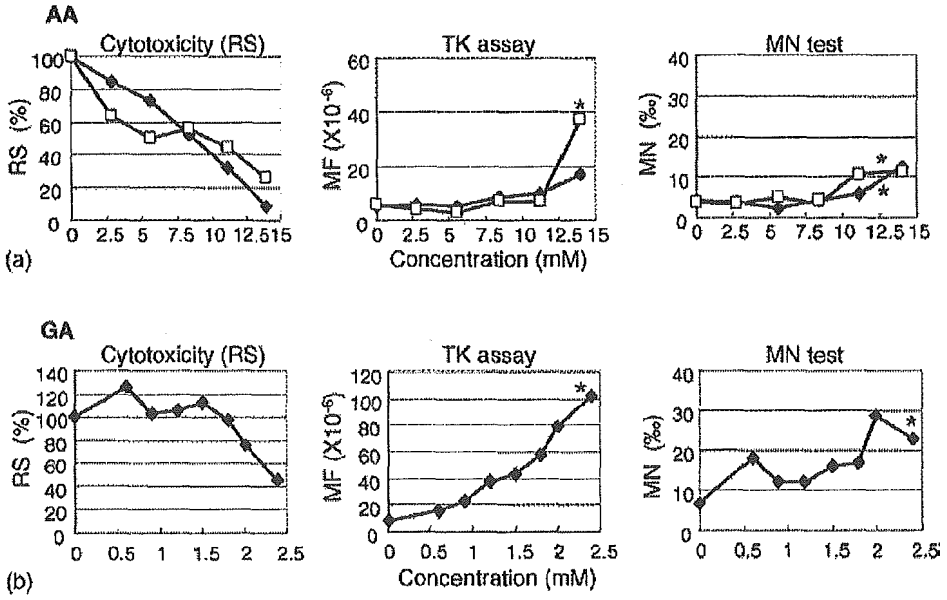

Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, respectively. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

Table 1
Cytotoxic and mutational responses to AA and GA, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) TK-mutants

Treatment	Cytotoxic and mutational response			LOH analysis at TK gene			
	RS (%)	MF ($\times 10^{-6}$)	% SG	No.	None LOH	Hemi-LOH	Homo-LOH
Vehicle [16]	100	2.19	56	56			
NG mutants				19	14(74)	3(16)	2(11)
SG mutants				37	0(0)	9(24)	28(76)
AA (14 mM, 4 h)	40	18.9	54	48			
NG mutants				22	11(50)	11(50)	0(0)
SG mutants				26	0(0)	13(50)	13(50)
GA (2.2 mM, 4 h)	12	55.5	36	44			
NG mutants				28	26(93)	2(7)	0(0)
SG mutants				16	0(0)	6(38)	10(62)

quencies at about four times the spontaneous level. We detected two distinct phenotypic classes of *TK* mutants in *TK* assay: NG and SG mutants. AA did not affect the proportion of SG mutants, while GA treatment lowered it (Table 1). This implies that GA induced primarily point mutations. In the COM assay, even at the highest concentration, AA did not induce DNA damage, while GA did so strongly starting at 0.6 mM (Fig. 2).

3.2. Molecular analysis of TK mutants

The *TK* mutants were independently isolated from the cells treated with 14 mM AA or 2.2 mM GA for 4 h. Table 1 shows the cytotoxicity (RS) and *TK* mutation frequency (MF) and proportion of SG mutants (% SG) by the treatment. Genomic DNA extracted from the mutants was subjected by the PCR-based LOH analysis to classify the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH) and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [13]. We analyzed 48 AA-induced and 44 GA-induced *TK* mutants and compared them to those of spontaneously occurring *TK* mutants described previously [16]. The fraction of hemi-LOH in AA-induced mutants, in which 50% each of NG and SG mutants exhibited hemi-LOH, was higher than in spontaneous mutants, indicating that AA-induced primarily deletions. GA, on the other hand, induced primarily NG mutants, and most (93%) of them were the non-LOH type, which is presumably generated by point and other small intragenic mutations. Among 16 GA-induced SG mutants, the percentages that were hemi-LOH (38%) and homo-LOH (62%) were similar to those observed in spontaneous SG mutants. Fig. 3 shows the mutation spectra of *TK* mutants found among treated and untreated TK6 cells. GA and ethyl methane sulfonate, an alkylating agent, produce similar spectra, as do AA and X-radiation.

Fig. 4 shows the distribution of LOH in AA-induced ($n = 37$), GA-induced ($n = 17$) and spontaneous ($n = 29$) LOH mutants. Because the majority of GA-induced mutants were the non-LOH type, we were able to map only 17 GA-induced LOH mutants. As a particular characteristic of AA-induced LOH mutants, we frequently observed small deletions limited to the *TK* locus. The

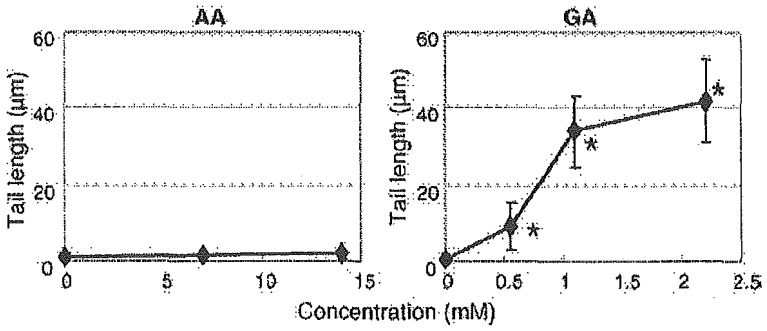

Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (*) statistically significant in the Dunnett's tests ($P < 0.05$).

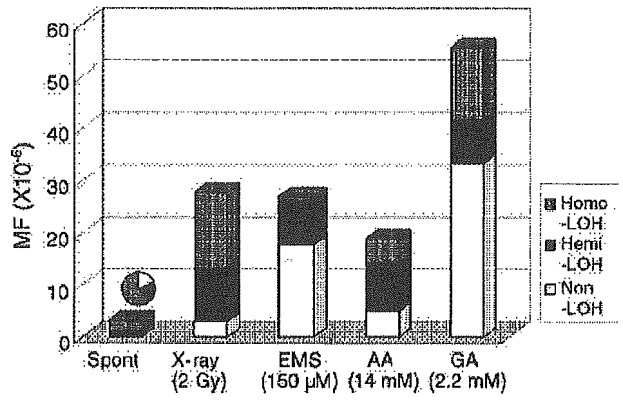

Fig. 3. Frequency and spectra of TK mutations in spontaneous and X-ray-induced (2 Gy), EMS-induced (150 μM, 4 h), AA-induced (14 mM, 4 h) and GA-induced (2.2 mM, 4 h) TK mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of normally growing (NG) and slowly growing (SG) mutants and the results of molecular analysis (Table 1). The data of spontaneous, X-ray-induced and EMS-induced mutation spectra were taken from our previous paper [13].

distribution of LOH in GA-induced and spontaneous LOH mutants was similar.

3.3. Cytotoxicity and genotoxicity of AA under metabolic activation

Rat liver S9 mix did not influence the cytotoxicity or genotoxicity of AA but it did enhance the activity of DBN, the positive control chemical (Fig. 5).

4. Discussion

A large number of studies about the in vitro genotoxicity of AA have been reported [8]. AA has consistently been negative in bacterial gene mutation assay in both the presence and absence of metabolic activation [23–25] but positive in chromosome aberration and sister chromatid exchange tests in Chinese hamster cell lines [24–26]. In mammalian cell assays, AA induces *Tk* but not *Hprt* gene mutations [24,25,27,28], and is negative in the COM assay even at high concentrations [27]. These results suggest that AA is clastogenic without directly damaging DNA. GA, on the other hand, is positive in most in vitro genotoxicity tests and is recognized as a mutagen [8,27,29]. In the present study, the higher concentrations of AA were positive in the MN and TK assay but negative in the comet assay. According to the in vitro genotoxicity test guideline, however, AA may be negative [30], because the guideline suggests that the maximum concentration should be 10 mM. Because the genotoxic responses at higher concentrations were reproducible, AA may be genotoxic, but its effect is very weak. GA, in contrast, was positive in all the assays, even under conditions of low cytotoxicity. These results are consistent with the reports described above.

The mammalian *TK* gene mutation assay can detect a wide range of genetic changes, including point mutations, small deletions, large-scale chromosomal deletions, inter-allelic recombination and aneuploidy, while

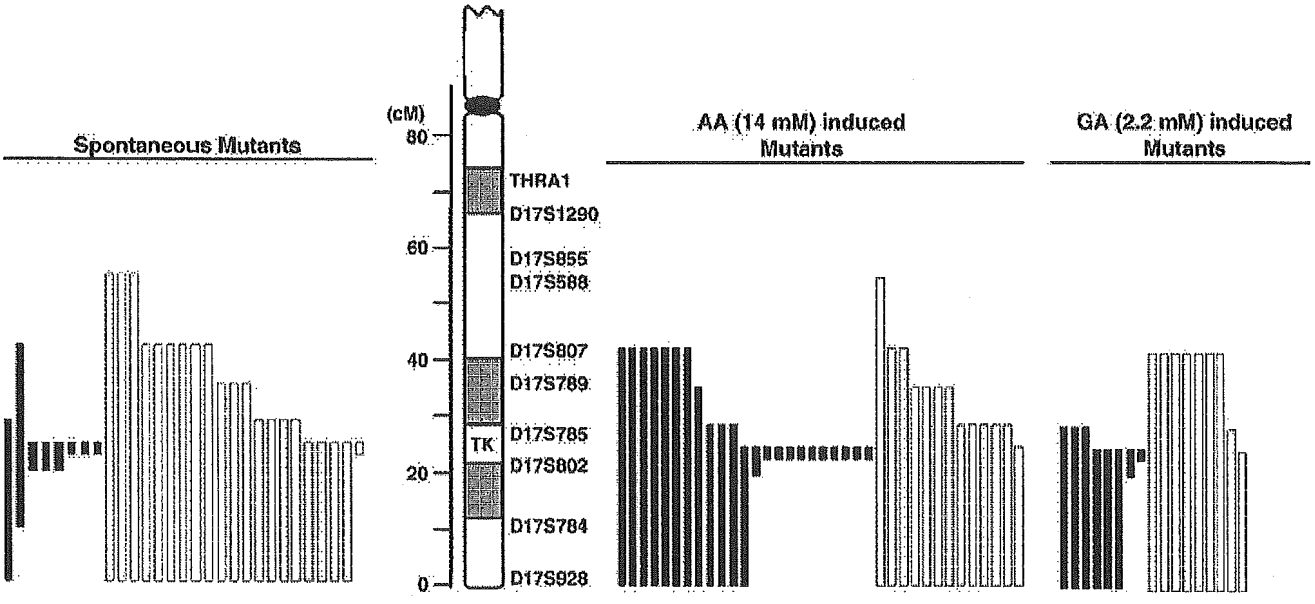

Fig. 4. The extent of LOH in spontaneous, AA-induced and GA-induced LOH mutants from TK6 cells. We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human TK locus maps to 17q23.2. Open and closed bars represent homo-LOH and hemi-LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 29 spontaneous mutants (10 NG and 19 SG mutants), 37 AA-induced mutants (11 NG and 26 SG) and 17 GA-induced mutants (2 NG and 15 SG). The data on spontaneous mutants were taken from our previous paper [13].

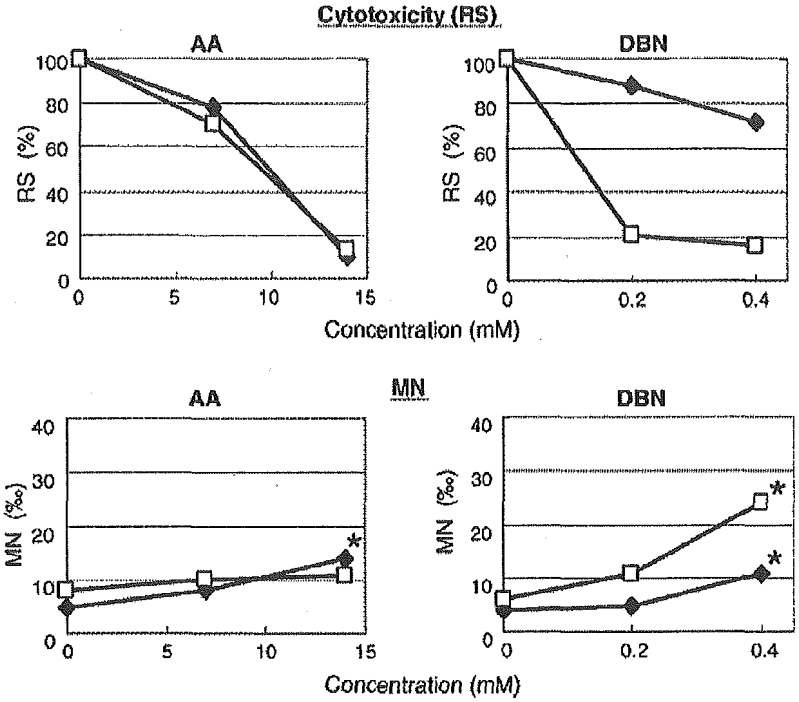

Fig. 5. Cytotoxicity (RS) and MN induction in TK6 cells treated with AA or DBN for 4 h in the presence (open symbol) or absence (closed symbol) of rat liver S9. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

the bacterial and mammalian *HPRT* gene mutation assays detect only point mutations and small intragenic deletions [13]. AA was positive only in the *TK* mutation assay, suggesting that AA causes predominantly large-scale chromosomal changes. Our molecular analysis of the *TK* mutants supported this hypothesis. The majority of the AA-induced *TK* mutants showed hemi-LOH, which is the result of a deletion, although the other types were also induced (Fig. 3). Deletions are thought to result from the repair of double strand breaks by non-homlogous end-joining [13]. Radiation-induced double strand breaks are repaired by non-homlogous end-joining, which leads to hemi-LOH. LOH-mapping analysis, however, revealed that AA frequently induces intermediate-sized deletions (100–3000 kb); the deletions encompass exons 4 and 7 of the *TK* locus but do not extend to the microsatellites loci of the vicinity. This type of deletion is rarely observed in radiation-inducing *TK* mutants [13]. Because the COM assay indicated that AA did not induce DNA damage, the deletion may not be caused by DNA damage directly. Mechanisms associated with global genomic instability should also be considered [10] because the LOH patterns, except for the intermediate-sized deletions, are generally similar to those observed in spontaneous mutants. Most GA-induced *TK* mutants, on the other hand, were the non-LOH type, as were most spontaneous ones, strongly supporting the positive results in bacterial gene mutation assay [29]. In contrast to AA, GA is a mutagen, inducing primarily point mutations.

AA is known to be metabolized to GA by CYP2E1 [9]. GA, an epoxide, forms adducts directly with DNA and protein, causing cytotoxicity and genotoxicity. GA forms mainly *N*7-(2-carbamoyl-2-hydroxyethyl) guanine and *N*3-(2-carbamoyl-2-hydroxyethyl) adenine and reacts with hemoglobin and cytoskeletal proteins [31–33]. Rat S9, however, did not affect AA cytotoxicity or genotoxicity, although it did enhance the cytotoxicity and genotoxicity of DBN, which is also metabolized by CYP2E1. This suggests that rat S9 does not work for activating AA. AA and GA are detoxified through glutathione conjugation, and GA is also detoxified by epoxy hydrolase (EH), which catalyzes the hydrolysis of GA to dihidroxy propionamide [34,35]. Other in vitro studies also failed to demonstrate the enhancement of AA genotoxicity by rat S9 [36,37]. Our results do not mean that AA is always detoxified rather than activated because DNA adducts are found in mice and rats given oral AA, and the genotoxicity of AA is consistently observed in in vivo studies [8,31,36,37]. Recently, Manjanatha et al. demonstrated in transgenic Big Blue™ mice that AA as well as GA induces endogenous *Hprt* and transgenic *cII* mutation at same level, and both chemicals cause predominantly base substitutions and frameshift mutations.

This result may indicate that AA is metabolized to GA in vivo [38]. Tests that use rat liver S9 for metabolic activation may not be appropriate for in vitro investigations of AA genotoxicity and metabolism. Transgenic cells expressing CYP2E1, however, would be useful for demonstrating the in vitro genotoxicity of AA [39].

In conclusion, AA is weakly genotoxic, causing chromosome aberrations and a type of genomic instability. GA, its epoxide metabolite, is highly reactive with DNA. GA is a strong mutagen, inducing predominantly point mutations, and it may contribute to human cancers.

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References

[1] IARC, Acrylamide, IARC Monographs on the Evaluation of Carcinogen Risk to Human: Some Industrial Chemicals, vol. 60, International Agency for Research on Cancer, Lyon, 1994, pp. 389–433.
[2] E. Tareke, P. Rydberg, P. Karlsson, S. Eriksson, M. Tornqvist, Acrylamide: a cooking carcinogen? Chem. Res. Toxicol. 13 (2000) 517–522.
[3] R.H. Stadler, I. Blank, N. Varga, F. Robert, J. Hau, P.A. Guy, M.C. Robert, S. Riediker, Acrylamide from Maillard reaction products, Nature 419 (2002) 449–450.
[4] d.S. Mottram, B.L. Wedzicha, A.T. Dodson, Acrylamide is formed in the Maillard reaction, Nature 419 (2002) 448–449.
[5] J.M. Rice, The carcinogenicity of acrylamide, Mutat. Res. 580 (2005) 3–20.
[6] E.J. Lehning, C.D. Balaban, J.F. Ross, M.A. Reid, R.M. LoPachin, Acrylamide neuropathy. I. Spatiotemporal characteristics of nerve cell damage in rat cerebellum, Neurotoxicology 23 (2002) 397–414.
[7] R.M. LoPachin, C.D. Balaban, J.F. Ross, Acrylamide axonopathy revisited, Toxicol. Appl. Pharmacol. 188 (2003) 135–153.
[8] K.L. Dearfield, G.R. Douglas, U.H. Ehling, M.M. Moore, G.A. Sega, D.J. Brusick, Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk, Mutat. Res. 330 (1995) 71–99.
[9] S.C. Sumner, T.R. Fennell, T.A. Moore, B. Chanas, F. Gonzalez, B.I. Ghanayem, Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice, Chem. Res. Toxicol. 12 (1999) 1110–1116.
[10] M. Friedman, Chemistry, biochemistry, and safety of acrylamide. A review, J. Agric. Food Chem. 51 (2003) 4504–4526.
[11] H.M. Bolt, Genotoxicity–threshold or not? Introduction of cases of industrial chemicals, Toxicol. Lett. 140–141 (2003) 43–51.
[12] H.L. Liber, W.G. Thilly, Mutation assay at the thymidine kinase locus in diploid human lymphoblasts, Mutat. Res. 94 (1982) 467–485.
[13] M. Honma, Generation of loss of heterozygosity and its dependency on p53 status in human lymphoblastoid cells, Environ. Mol. Mutagen. 45 (2005) 162–176.
[14] 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.
[15] K. Sekihashi, H. Saitoh, Y. Sasaki, Genotoxicity studies of stevia extract and steviol by the comet assay, J. Toxicol. Sci. 27 (Suppl. 1) (2002) 1–8.
[16] M. Watanabe-Akanuma, T. Ohta, Y.F. Sasaki, A novel aspect of thiabendasole as a photomutagen in bacteria and cultured human cells, Mutat. Res. 158 (2005) 213–219.
[17] L. Zhan, H. Sakamoto, M. Sakuraba, D.S. Wu, L.S. Zhang, T. Suzuki, M. Hayashi, M. Honma, Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells, Mutat. Res. 557 (2004) 1–6.
[18] T. Matsushima, M. Hayashi, A. Matsuoka, M. Ishidate Jr., K.F. Miura, H. Shimizu, Y. Suzuki, K. Morimoto, H. Ogura, K. Mure, K. Koshi, T. Sofuni, Validation study of the in vitro micronuclei test in a Chinese hamuster lung cell line (CHL/IU), Mutagenesis 14 (1999) 569–580.
[19] S.A. Amundson, H.L. Liber, A comparison of induced mutation at homologous alleles of the tk locus in human cells, Mutat. Res. 247 (1991) 19–27.
[20] 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.
[21] T. Omori, M. Honma, M. Hayashi, Y. Honda, I. Yoshimura, A new statistical method for evaluating of L5178Ytk± mammalian cell data using microwell method, Mutat. Res. 517 (2002) 199–208.
[22] 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. Carcinog. 28 (2000) 203–214.
[23] E. Zeiger, B. Anderson, S. Haworth, T. Lawlor, K. Mortelmans, W. Speck, Salmonella mutagenicity tests: III. Results from the testing of 255 chemicals, Environ. Mutagen. 9 (Suppl. 9) (1987) 1–109.
[24] H. Tsuda, C.S. Shimizu, M.K. Taketomi, M.M. Hasegawa, A. Hamada, K.M. Kawata, N. Inui, Acrylamide; induction of DNA damage, chromosomal aberrations and cell transformation without gene mutations, Mutagenesis 8 (1993) 23–29.
[25] A.G. Knaap, P.G. Kramers, C.E. Voogd, W.G. Bergkamp, M.G. Groot, P.G. Langebroek, H.C. Mout, J.J. van der Stel, H.W. Verharen, Mutagenic activity of acrylamide in eukaryotic systems but not in bacteria, Mutagenesis 3 (1988) 263–268.
[26] T. Sofuni, M. Hayashi, A. Matsuoka, M. Sawada, Mutagenicity tests on organic chemical concominants in city water and related compounds. II. Chromosome aberration tests in cultured mammalian cells, Eisei Shiken. Hok. 103 (1985) 64–75.
[27] M. Baum, E. Fauth, S. Fritzen, A. Herrmann, P. Mertes, K. Merz, M. Rudolphi, H. Zankl, G. Eisenbrand, Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood, Mutat. Res. 580 (2005) 61–69.
[28] M.M. Moore, A. Amtower, C. Doerr, K.H. Brock, K.L. Dearfield, Mutagenicity and clastogenicity of acrylamide in L5178Y mouse lymphoma cells, Environ. Mutagen. 9 (1987) 261–267.
[29] K. Hashimoto, H. Tanii, Mutagenicity of acrylamide and its analogues in *Salmonella typhimurium*, Mutat. Res. 158 (1985) 129–133.
[30] C.S. Aaron, G. Bolcsfoldi, H.R. Glatt, M. Moore, Y. Nishi, L. Stankowski, Theiss F J., E. Thompson, Mammalian cell gene mutation assays working group report, Mutat Res. 312 (1994) 235–239.

[31] d.C. Gamboa, M.I. Churchwell, L.P. Hamilton, L.S. Von Tungeln, F.A. Beland, M.M. Marques, D.R. Doerge, DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice, Chem. Res. Toxicol. 16 (2003) 1328–1337.
[32] D.M. Lapadula, M. Bowe, C.D. Carrington, L. Dulak, M. Friedman, M.B. Abou-Donia, In vitro binding of [14C]acrylamide to neurofilament and microtubule proteins of rats, Brain Res. 481 (1989) 157–161.
[33] S.C. Sumner, C.C. Williams, R.W. Snyder, W.L. Krol, B. Asgharian, T.R. Fennell, Acrylamide: a comparison of metabolism and hemoglobin adducts in rodents following dermal, intraperitoneal, oral, or inhalation exposure, Toxicol. Sci. 75 (2003) 260–270.
[34] B. Paulsson, A. Rannug, A.P. Henderson, B.T. Golding, M. Tornqvist, M. Warholm, In vitro studies of the influence of glutathione transferases and epoxide hydrolase on the detoxification of acrylamide and glycidamide in blood, Mutat. Res. 580 (2005) 53–59.
[35] S.C. Sumner, L. Selvaraj, S.K. Nauhaus, T.R. Fennell, Urinary metabolites from F344 rats and B6C3F1 mice coadministered acrylamide and acrylonitrile for 1 or 5 days, Chem. Res. Toxicol. 10 (1997) 1152–1160.
[36] A. Besaratinia, G.P. Pfeifer, Genotoxicity of acrylamide and glycidamide, J. Natl. Cancer Inst. 96 (2004) 1023–1029.
[37] A. Besaratinia, G.P. Pfeifer, DNA adduction and mutagenic properties of acrylamide, Mutat. Res. 580 (2005) 31–40.
[38] M.G. Manjanatha, A. Aidoo, S.D. Shelton, M.E. Bishop, L.P. MacDaniel, D.R. Doerge, Genotoxicity of acrylamide and its metabolite glycidamide administrated in drinking water to male and female Big Blue mice, Environ. Mol. Mutagen, in press (Epub ahead of prints).
[39] H. Glatt, H. Schneider, Y. Liu, V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals, Mutat. Res. 580 (2005) 41–52.

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原　著

無機および有機ヒ素化合物のin vitro遺伝子突然変異誘発性と，その食物摂取からの遺伝毒性リスク

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In vitro genotoxicity of inorganic and organic arsenics and their genotoxic risk through food intake

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Summary

Arsenic compounds contained in sea foods have raised public health concerns, because their chronic exposure through dietary intake may increase cancer risk. In the present study, we investigated the in vitro genotoxicity of two inorganic arsenics (arsenite; As[III], arsenate; As[V]) and three organic arsenics (monomethylarsonic acid; MMAA, dimethylarsenic acid; DMAA, trimethylarsine oxide; TMAO) using mouse lymphoma *Tk* assay (MLA). In the standard MLA with 3 h treatment, exposure to As[III] and As[V] significantly induced *Tk*-mutants. The genotoxicity of As[III] was over 50-times greater than that of As[V]. Among organic arsenics, on the other hand, only DMAA showed weak genotoxicity with 3 h treatment at high doses. In the 24 h treatment MLA, DMAA and TMAO weakly induced *Tk*-mutants. These results indicate that inorganic arsenics rather than organic arsenics should be considered for genotoxic risk. We discussed the genotoxic risk of arsenic compounds through dietary intake.

Keywords: arsenite[III], arsenate[V], organic arsenics, mouse lymphoma *Tk* assay (MLA), genotoxic risk

緒　言

ヒ素およびその化合物は，かつては毒薬として用いられた経緯もあり，人体に非常に有害であることはよく知られている（ATSDR, 2000）．飲み込んだ際の急性症状は，吐き気，嘔吐，下痢，激しい腹痛などが見られ，場合によってショック状態から死に至る．我が国には1955年に粉ミルクの製造過程にヒ素が混入し，それを飲んだ1万人以上の乳幼児が中毒を起こし，138名の死者を出した痛ましい事件がある（森永ヒ素ミルク事件）．慢性症状としては，剥離性の皮膚炎や色素沈着，骨髄障害，末梢性神経炎，黄疸，腎不全などがあげられる．疫学的研究からヒ素およびヒ素化合物は，発がん性も指摘されており，WHOのがん研究機構（IARC）ではヒ素をGroup 1（人に対して発がん性あり）に分類している（IARC, 1987）．

ヒ素は自然にも存在しており，その管理が難しい．水

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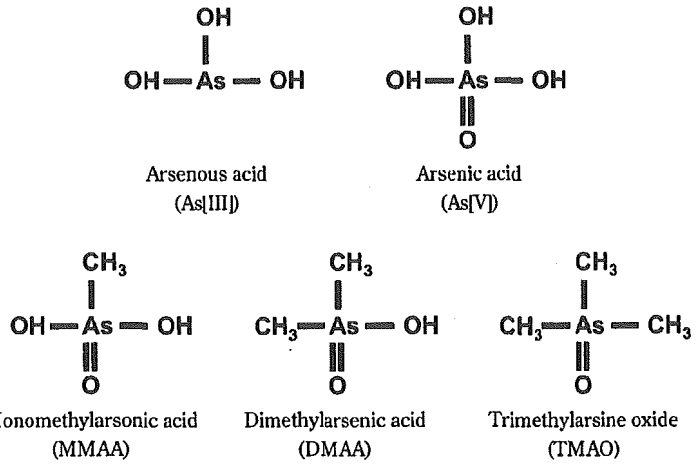

Fig. 1 Inorganic and organic arsenic compounds in marine organisms

道水中のヒ素濃度はWHOの飲料水水質ガイドラインに従い，発がんリスクをできるだけ低くするように管理されている（WHO, 2004）．また，ヒ素化合物は，生体内にごく微量が存在しており，人体にとって微量必須元素であると考えられている．ただし，生体内のヒ素の大部分は代謝により毒性の低い有機ヒ素化合物として存在している（ATSDR, 2000）．

2004年7月28日，英国食糧規格庁（Food Standard Agency）は，海草のひじき（Hijiki）に無機ヒ素（Inorganic arsenic）が大量に含まれているという調査結果に基づき，国民に対してひじきを食べないように勧告した（FSA, 2004）．海産食品にはこれまでヒ素化合物が多く含まれていることが報告されている．特に，ひじき，こんぶなどの海草類，えびなどの甲殻類，カレイなどの魚類は，ヒ素含有量が高いことが知られている（Fukui et al., 1981）．これら食品中からのヒ素化合物の摂取をできるだけ低レベルに抑えることが，水道水の管理と同様に，一般市民の発がんリスクを減らすことになるのかもしれない．

海産物中に含まれるヒ素化合物には，最も毒性が高いとされている三価の無機ヒ素（As[III]）だけでなく，五価の無機ヒ素（As[V]）や，有機ヒ素化合物などがある．そして，これらの含有量は海産物の種類によって異なることが知られている（Fukui et al., 1981）．また，これらヒ素化合物の毒性はその化学形態によって大きく異なる（ATSDR, 2000）．本研究では，2つの無機ヒ素化合物（As[III], As[V]）と，比較的海産物に多く含まれている3つの有機ヒ素化合物（モノメチルアルソン酸：monomethylarsonic acid（MMAA），ジメチルアルシン酸：dimethylarsenic acid（DMAA），トリメチルアルシンオキシド：trimethylarsine oxide（TMAO））（Fig. 1）の遺伝毒性誘発性をマウスリンフォーマ*Tk*試験（MLA）によって評価した．また，これらヒ素化合物の摂取量と，遺伝毒性の程度を，他の食品中から摂取する可能性のある化学物質のそれらと比較し，食品中からのヒ素化合物の遺伝毒性リスクを考察した．

実験材料および方法

1. 試験検体

亜ヒ酸ナトリウム（sodium arsenite（As[III]）；Cas. No. 7784-46-5, MW130）は，関東化学工業製，ヒ酸二ナトリウム（sodium arsenate（As[V]）；CAS. No. 7778-43-0, MW186）は，和光純薬工業製，特級試薬を用いた．モノメチルアルソン酸（monomethylarsonic acid（MMAA）；CAS. No. 124-58-3, MW140），ジメチルアルシン酸（dimethylarsenic acid（DMAA）；CAS. No. 75-60-5, MW138），トリメチルアルシンオキシド（trimethylarsine oxide（TMAO）；CAS. No. 4964-14-1, MW136）は，トリケミカル研究所製の純度99.9%のものを用いた．

2. 用量設定試験

細胞を試験検体で一定時間処理し，その後の48時間における細胞増殖性を，細胞数を計測して求め，陰性対照と比較した（Relative Suspension Growth; RSG）．陰性対照の10～20%になる濃度を最高用量として設定した．

3. マウスリンフォーマ*Tk*試験（MLA）

MLAはマイクロウェル法で行い，プロトコールはHonmaらの方法に従った（Honma et al., 1999a）．S9非存在下で，細胞を試験検体で3時間処理し，48時間の発現時間をおいて，*Tk*突然変異検出のために細胞をプレーティングした．細胞毒性の指標としては処理直後の相対生存率（Relative Survival; RS）と，処理後の増殖性と生存率を考慮した値（Relative Total Growth; RTG）を用いた．有機ヒ素化合物に関しては，24時間処理を実施した．24時間処理のプロトコールは，Honmaらの方法に従った（Honma et al., 1999b）．

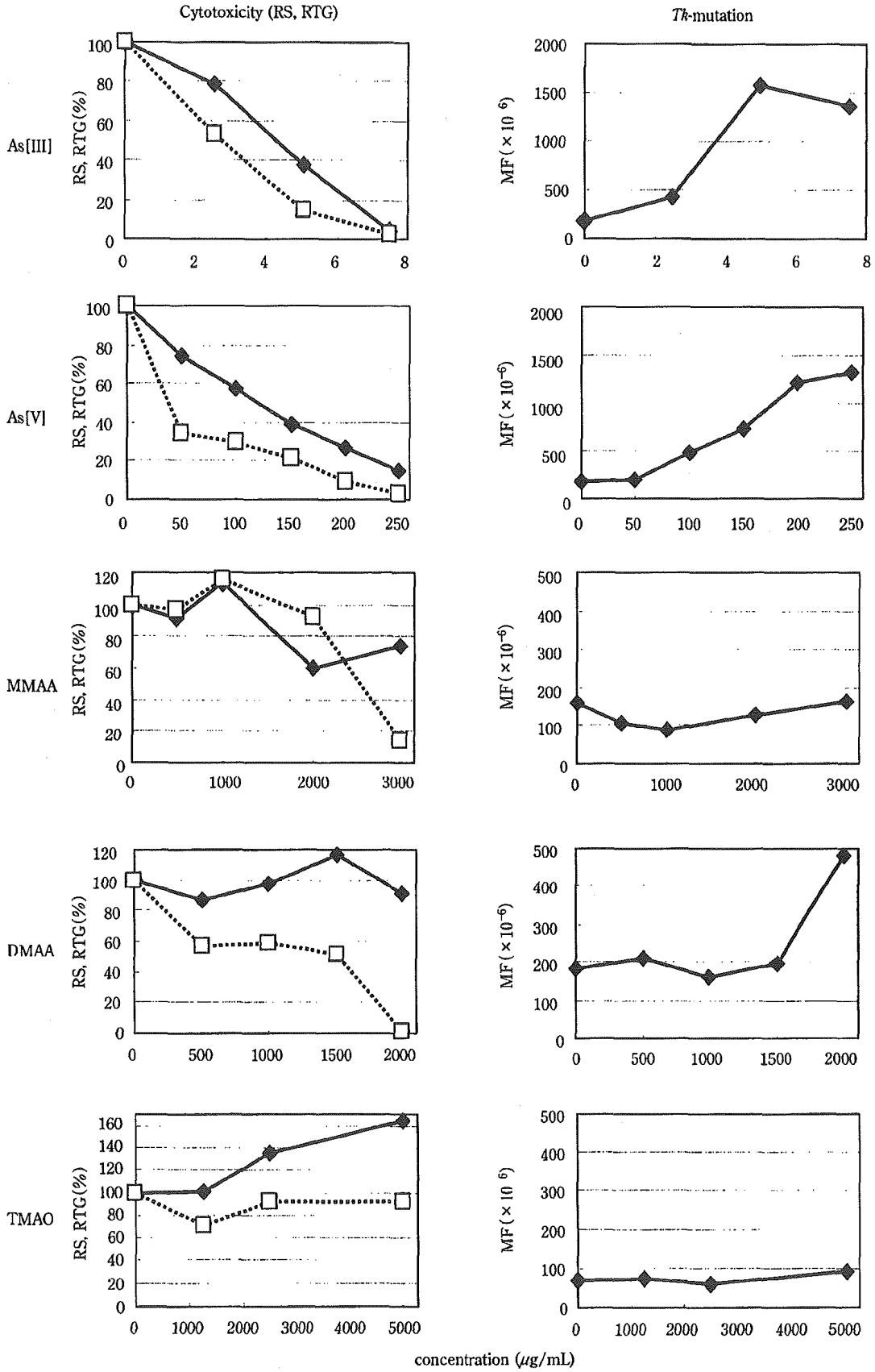

Fig. 2 Cytotoxicity (relative survival; RS, relative total growth; RTG) and *Tk*-mutation frequency (MF) in MLA treated with As[III], As[V], MMAA, DMAA, and TMAO for 3 h. In cytotoxicity, closed symbol is RS, and open symbol is RTG.

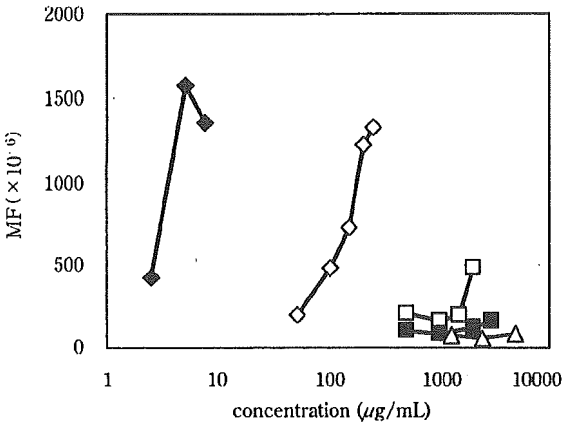

Fig. 3 Comparison of *Tk*-mutation frequencies (MF) in 3 h treatment test treated with As[III]◆, As[V]◇, MMAA■, DMAA□, and TMAO△.

4. 統計的解析

MLAのデータはOmoriらの統計的手法により，陽性，陰性を判定した（Omori et al., 2002）．この方法はDunnett法による陰性対照との比較検定，Simpson-Margolin法による用量依存的な傾向性検定を組み合わせた方法である．

結　　果

1. 用量設定試験

細胞を試験検体で3時間処理し，陰性対照の10～20%のRSGを示す濃度を本試験における最高用量として設定した（data not shown）．この試験より，As[III]: 10 μg/mL，As[V]: 250 μg/mL，MMAA: 3000 μg/mL，DMAA: 2500 μg/mL，TMAO: 5000 μg/mLを最高用量として設定した．TMAOは5000 μg/mLにおいてもほとんど細胞毒性を示さなかったがMLAの試験ガイドラインに従い，5000 μg/mLを最高用量とした．

2. 3時間処理試験

3時間処理によるMLAの結果をFig. 2に示す．無機ヒ素化合物のAs[III]，As[V]は用量依存的に細胞毒性（RS，RTG）を示し，それに伴い高い突然変異の誘発が認められた．統計学的にも突然変異頻度の増加は有意であった（有意水準1%）．As[III]の5 μg/mL，As[V]の250 μg/mLで陰性対照の15倍の突然変異の誘発が観察された．MLAでは，2種類の変異体（small colony mutant; SC，large colony mutant; LC）が観察されるが，これらヒ素化合物によって誘発された変異体のSC/LC比は特に陰性対照との違いは認められなかった（data not shown）．As[III]の最高用量の10 μg/mLではRS，RTGとも毒性が高すぎたため突然変異のデータは得られなかった．突然変異頻度を2倍増加させる用量（Double Mutation Frequency Dose; DMFD）はAs[III]では0.78 μg/mL，As[V]では39.5 μg/mLと計算できた．このことからAs[III]はAs[V]に比べて，用量当たり約50倍程度の遺伝毒性を持つ．

一方，有機ヒ素化合物であるMMAA，TMAOは試験濃度では全く突然変異の誘発を示さなかった．特にTMAOは細胞毒性も全く示さなかったことから，極めて毒性の低い化合物であることが予想された．DMAAは2000 μg/mLの高濃度で突然変異の増加が見られ，統計学的には有意に陽性であったが（有意水準5%），RTGが1%であり，強い細胞毒性による非特異的反応とも考えられる．2500 μg/mLでは，毒性が高すぎたため突然変異のデータは得られなかった．5つのヒ素化合物の突然変異誘発頻度の比較をFig. 3に示した．

DMAAは細胞毒性の指標であるRTGとRSに大きな差が見られた．RTGがRSに比べて低いことは強い細胞増殖阻害が起きていることを予想させる．

3. 24時間処理試験

3種類の有機ヒ素化合物は短時間処理において，無機ヒ素化合物と比較して，強い突然変異誘発性を示さなかったことから，24時間処理を実施した．3時間処理と同様に用量設定試験を実施し，最高用量を設定し，本試験を行った．本試験の結果をFig. 4に示す．全ての有機ヒ素化合物は用量依存的に細胞毒性を示した．一方，突然変異誘発性に関して，MMAAは陰性であったが，DMAA，TMAOは統計学的には陽性を示した（有意水準5%）．ただし，これら陽性反応も，RTG 5%以下の強い毒性下での反応であり，非特異的な影響であるのかもしれない．3つの有機ヒ素化合物の突然変異誘発頻度の比較をFig. 5に示した．

考　　察

1. 遺伝毒性ハザードとしてのヒ素化合物

無機ヒ素化合物の中でも毒性の高いAs[III]に関しては，多くの遺伝毒性の報告がある（Gradecka et al., 2001）．In vitro遺伝毒性においては，As[III]はエームス試験陰性を示すのに対して，培養細胞を用いた染色体異常試験，姉妹染色分体交換試験，コメット試験では陽性を示す（Basu et al., 2001; Gebel, 2001）．マウスを用いたin vivo試験では，染色体異常，小核の誘発が報告されている（Gebel, 2001）．このように，染色体レベルの強い遺伝毒性や，DNA損傷性が多数報告されているにもかかわらず，エームス試験では陰性であることから，As[III]は点突然変異を誘発するようなmutagenではなく，染色体レベルの損傷を引き起こすclastogenであるとされている（Gebel, 2001）．MLAは常染色体劣性型の遺伝子突然変異試験であり，点突然変異から，染色体レベルの変異までを検出できる広域スペクトルを持つ突然変異検出系である（Honma et al., 2001）．今回の我々の

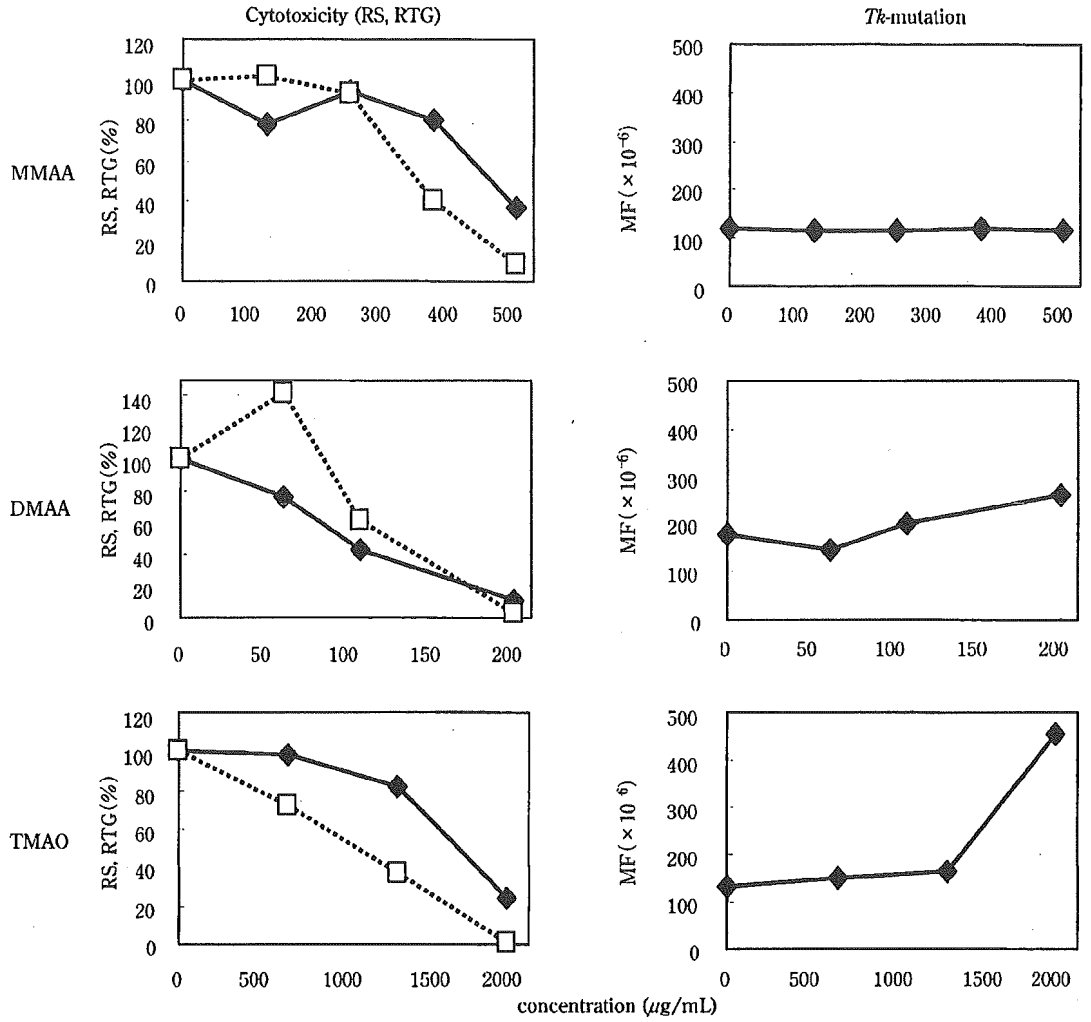

Fig. 4 Cytotoxicity (relative survival; RS, relative total growth; RTG) and *Tk*-mutation frequency (MF) in MLA treated with As MMAA, DMAA, and TMAO for 24 h. In cytotoxicity, closed symbol is RS, and open symbol is RTG.

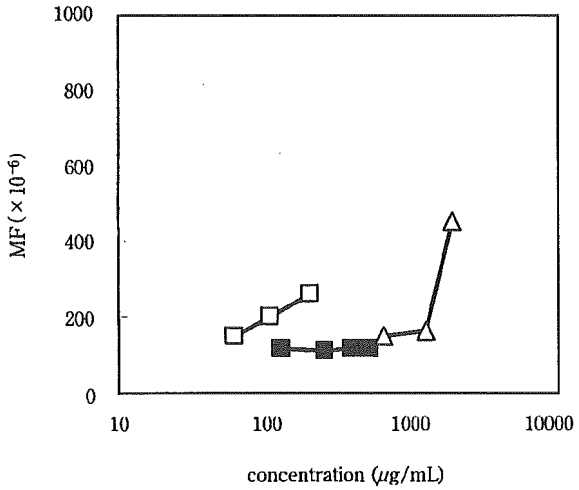

Fig. 5 Comparison of *Tk*-mutation frequencies (MF) in 24 h treatment test treated with MMAA-■-, DMAA-□-, and TMAO-△-.

研究でAs[III]がMLAで強い遺伝子突然変異誘発性を示したことは，As[III]が染色体レベルの遺伝子突然変異を引き起こすことを示唆している．MooreらもMLAでAs[III]とAs[V]の突然変異誘発性を報告している(Moore et al., 1997)．我々の結果と同様に，As[III]は，As[V]の1/10以下の試験用量で高い突然変異誘発性を示すことを報告している．

有機ヒ素化合物の遺伝毒性についての報告は多くない(Gebel, 2001; Kaise et al., 1996)．今回試験した3つの有機ヒ素化合物MMAA，DMAA，TMAOは，無機ヒ素化合物が生体内に取り込まれた後の主たる代謝産物であり，人体や，多くの食物中に存在が確認されている．解毒経路の代謝産物であり，その毒性は無機ヒ素化合物に比べて極めて低い．In vivo遺伝毒性試験において，これら有機ヒ素化合物の突然変異誘発性は陰性と報告されている（Noda et al., 2002)．今回のMLAでは，有機ヒ素化合物3種の中で3時間処理，24時間処理とも突然変異

Table 1 Human Exposure/Rodent Potency (HERP) and Human Exposure/Genotoxic Potency (HEGEP)

Compounds (Major origin)	IARC	Average daily intake (μg/day)*	HERP*	Genotoxicity			
				DMFD (μg/mL)	HEGEP	Cell, Cond.	Ref
Saccharin (Artificial sweetener)	3	95000	0.06	12000	7.9	MLA	Clive et al., 1979
Dimehtylnitrosamine (Beer)	2A	0.646	0.01	0.07	9.2	MLA, rat S9	McGregor et al., 1988
Acrylamide (Potato chips et al.)	2A	40	0.01	100	0.4	WTK-1	Unpublished data
Aflatoxin B1 (Peanut et al.)	1	0.018	0.008	0.0075	2.4	MLA, rat S9	Preisler et al., 2000
AF-2 (Preservative, -1975)	2B	4.8	0.0002	2.5	1.92	WTK-1	Unpublished data
IQ (Burnt foods)	2A	0.0064	0.00001	7.2	0.89	WTK-1	Unpublished data
Kojic acid (Miso, soy source)	2B	0.2	0.0000005	2500	0.00008	WTK-1, rat S9	Unpublished data
As[III] (Hijiki, cooked)	1	1.6	-	0.78	2.05	MLA	Present study
As[V] (Hijiki, cooked)	1	4.4	-	39.5	0.11	MLA	Present study
As[III] (Tap water and other natural sources, Max)	1	107	-	0.78	137.2	MLA	Present study

* Data from Gold et al. (2002)

誘発性が認められたのはDMAAのみであった．また，3時間処理試験での細胞毒性試験からDMAAは強い細胞増殖阻害作用を持つことが示唆された．Kashiwadaらはマウスに DMAAを腹腔内投与後，骨髄細胞において，M期での細胞周期の停止，および異数染色体細胞の増加が観察されたことを報告している（Kashiwada et al., 1998）．このような現象はコルヒチンなどの細胞分裂毒の投与で，高頻度に観察されることから，DMAAは細胞分裂毒様の作用を持つことを予想させる．MLAは細胞分裂毒による染色体の異数化を介した，染色体の脱落による突然変異も検出することができる（Honma et al., 2001）．DMAAの持つ弱い遺伝子突然変異誘発性は，このような細胞分裂毒様の作用が関係しているのかもしれない．TMAOでもRSとRTGに差が見られた．24時間処理でTMAOがわずかに突然変異誘発性を示したのは，TMAOも弱い細胞分裂毒様の作用を持つことを示しているのかもしれない．

Mooreらは，MMAA，DMAAについてもMLAを実施し，両化合物とも3000 μg/mL以上の高濃度で，弱い突然変異誘発性を認めている（Moore et al., 1997）．MMAAの結果は我々の今回の結果と異なるものであるが，試験用量や，誘発性を考慮すると，その強さは大きくはない．

これらの結果から，無機化合物であるAs[III]とAs[V]は遺伝毒性物質であるが，有機ヒ素化合物であるMMAA，DMAA，TMAOの遺伝毒性はないか，あったとしても極めて弱いものと判断できる．

2. 遺伝毒性リスクとしてのヒ素化合物

ヒ素は齧歯類を用いた発がん性試験では陰性を示すが，疫学的研究から発がん性が明らかであるため，IARCではGroup I（人に対して発がん性あり）に分類されている（IARC, 1987）．ヒ素は我々の環境中に普遍的に存在する．そのため水道水中のヒ素濃度は，発がんリスクをできるだけ低減化させるため管理されている．現在，水道水中のヒ素濃度の基準は0.01 mg/Lである（WHO, 2004）．この濃度の発がん率は10万人中60人であり，他の環境発がん物質の基準の10倍以上も高い．これは，ヒ素が自然由来であり，その管理が困難であるためのやむを得ない措置と言える．

2004年7月28日に英国食糧規格庁（Food Standard Agency）は，海草のひじき（Hijiki）に無機ヒ素化合物が大量に含まれている理由で，国民に対してひじきを食べないように勧告した（FSA, 2004）．ひじきには乾燥重量当たり総ヒ素量として36～80 μg/gのヒ素化合物が含まれており（鴨志田ら，2005），水抽出物からの成分分析により，その83%が無機ヒ素化合物と報告されている（As[III]；22%，As[V]；61%）（Fukui et al., 1981）．他の多くの海産物にもヒ素が含まれるが，無機ヒ素の含有量はひじきが圧倒的に高い．しかし，ひじきは通常の調理過程（水戻し，加熱）で，約90%のヒ素が流出することが知られている（鴨志田ら，2005）．日本人のひ

じきの1日平均消費量は0.9 gであり（MHLW, 2004），仮に80 μg/gのヒ素を含むひじきを調理し，食したとすると，1日当たり約6 μgの無機ヒ素を摂取する計算になる（As[III]；1.6 μg，As[V]；4.4 μg）．WHOが定めた無機ヒ素のPTWI（暫定的耐用週間摂取量）は15 μg/kg/weekであるが，これは体重50 kgの人で，1日当たり107 μgのヒ素に相当する．このことから，日本人平均の18倍以上のひじきを食さない限り，PTWIを超えることはない．従って，バランスのよい食生活を心がければ，ひじきによる健康上のリスクは高まることがないと考えられる．

日常生活において摂取が避けられない発がん物質のリスク評価として，GoldらはHuman Exposure/Rodent Potency Index; HERPの利用を提唱している（Gold et al., 1990）．これは，人がその発がん物質暴露する体重当たりの平均暴露量を，その物質が齧歯類を用いた発がん性試験において動物の半数にがんを引き起こす量（TD50）で割ったものである．従って，前者が大きければ大きいほど，後者が小さければ小さいほど，HERPの値は大きくなり，そのリスクも高いと判断される．いくつかの例をTable 1に示した．HERP値の絶対量は1を基準として，それより高いものは，人での暴露が，動物実験でのTD50を引き起こす暴露量を超えることを示唆しており，また，相対的な数値は日常生活における発がんリスクのランキングを示す．しかしながら，HERP値はTD50を基礎としているため，齧歯類発がん性試験において陰性を示す物質については適用できない．実際に海産物から摂取されるヒ素のHERP値は計算されていない．この場合，齧歯類発がん性試験に替わる定量的データとして，遺伝毒性試験データを使い，HERPと同様にHuman Exposure/Genotoxic Potency; HEGEPを算出することを提案したい．ここでは，人がその発がん物質暴露する平均1日暴露量（μg/day）を，MLA，もしくはそれに準ずる遺伝子突然変異試験において，その発がん物質暴露が2倍の突然変異誘発率を引き起こす濃度（Double Mutation Frequency Dose; DMFD（μg/mL））で割ったものである．HEGEP値はHERPと異なり，その絶対値は生物学的な意味を持たない．また，in vitro試験データと，人の平均1日暴露量（μg/day）を組み合わせることは，個々の物質の遺伝毒性の発現メカニズムを無視した方法である．しかしながら，多くの遺伝毒性物質のHEGEPを，HERPと同様にランキングすることにより，他の遺伝毒性物質との相対的リスクをある程度理解することには有効であると考えられる．HEGEPの計算には，定量的な遺伝毒性試験パラメータであれば何でも利用でき，たとえば，染色体異常試験のD20値を基礎としたHEGEPも算出可能である．HEGEPの計算例もTable 1に示した．ここでは，MLA，もしくはそれと同程度の検出感度を持つWTK-1細胞による*Tk*突然変異試験でのDMFDを定量パラメータとしてHEGEPを計算した．

調理されたひじきから摂取される無機ヒ素量は先の計算からAs[III]が1.6 μg，As[V]が4.4 μgと算出できる．毒性が高いAs[III]のHEGEPは2.05であり，この遺伝毒性リスクはピーナッツ等からのaflatoxin B1（2.4）や，焦げた食品からのIQ（0.89）とほぼ同程度であり，日常生活において特に際立って遺伝毒性のリスクを増加させるとは考えられない．また，水道水等から日常生活において，別に70倍近くものAs[III]を摂取している可能性があり，それを考慮しても，ひじきから摂取しうるAs[III]の遺伝毒性リスクは，日常生活を極端に脅かすものではないと考えられる．

このように，日常生活中に受ける可能性のある遺伝毒性リスクをHEGEPとして算出し，他の物質と相対的に評価することは，そのリスクを理解しやすい．HERPとHEGEPの値を考慮し，発がん性，遺伝毒性リスクを総合的に評価することは極めて現実的な手法と考えられる．

結　論

ヒ素化合物の中で，無機化合物であるAs[III]，As[V]は，明らかに遺伝毒性物質である．ひじき中にはこれら無機ヒ素化合物が比較的多く含まれているが，その平均摂取量，および水道水等の他の摂取要因のレベルを考慮すると，ひじき食を介して摂取するヒ素化合物の遺伝毒性リスクは大きくないものと考えられる．

参考文献

ATSDR; Agency for Toxic Substances and Disease Registry (2000) Arsenic Toxicity Case Studies in Environmental Medicine. (http://atsdr1.atsdr.cdc.gov/HEC/CSEM/arsenic/arsenic.pdf)

Basu, A., J. Mahata, S. Gupta and A.K. Giri (2001) Genetic toxicology of a paradoxical human carcinogen, arsenic: a review, Mutat.Res., 488, 171-194.

Clive, D., K.O. Johnson, J.F. Spector, A.G. Batson and M.M. Brown (1979) Validation and characterization of the L5178Y/$TK^{+/}$ mouse lymphoma mutagen assay system, Mutat. Res., 59, 61-108.

FSA; Food Standard Agency (2004) Agency advises against eating hijiki seaweed. (http://www.food.gov.uk/news/pressreleases/2004/jul/hijikipr)

Fukui, S., T. Hirayama, M. Nohara and Y. Sakagami (1981) Studies on the chemical forms of arsenic in some sea foods and in urine after ingestion of these foods, Shokuhin Eiseigaku Zasshi, 22, 513-519.

Gebel, T.W. (2001) Genotoxicity of arsenical compounds, Int. J. Hyg. Environ. Health, 203, 249-262.

Gold, L.S., L. Bernstein and B.N. Ames (1990) The importance of ranking possible carcinogenic hazards using HERP, Risk Anal., 10, 625-628.

Gold, L.S., T.H. Slone, N.B. Manley and B.N. Ames (2002) Misconceptions about the Causes of Cancer, Risk Controversy Series, The Fraser Institute, Vancouver, Canada.

Gradecka, D., J. Palus and W. Wasowicz (2001) Selected mechanisms of genotoxic effects of inorganic arsenic compounds, Int. J. Occup. Med. Environ. Health, 14, 317-328.

Honma, M., M. Hayashi, H. Shimada, N. Tanaka, S. Wakuri, T. Awogi, K.I. Yamamoto, N. Kodani, Y. Nishi, M. Nakadate and T. Sofuni (1999a) Evaluation of the mouse lymphoma *Tk* assay (microwell method) as an alternative to the in vitro chromosomal aberration test, Mutagenesis, 14, 5-22.

Honma, M., L.Z. Zhang, H. Sakamoto, M. Ozaki, K. Takeshita, M. Momose, M. Hayashi and T. Sofuni (1999b) The need for long-term treatment in the mouse lymphoma assay, Mutagenesis, 14, 23-29.

Honma, M., M. Momose, H. Sakamoto, T. Sofuni and M. Hayashi (2001) Spindle poisons induce allelic loss in mouse lymphoma cells through mitotic non-disjunction, Mutat. Res., 493, 101-114.

IARC; International Agency for Research on Cancer (1987) Arsenic and arsenic compounds, IARC monographs supplement, 7, 100-106.

Kaise, T., Y. Oya-Ohta, T. Ochi, T. Okubo, K. Hanaoka, K. Irgolic, T. Sakurai and T. Matsubara (1996) Toxicological study of organic arsenic compound in marine algae using mammalian cell culture technique, Shokuhin Eiseigaku Zasshi, 37, 135-141.

鴨志田道子，貝瀬利一，花岡研一，長岡(浜野) 恵，米谷民雄，北浜裕司，西村光正 (2005) 調理過程におけるひじき中ヒ素の減衰，第89回日本食品衛生学会学術講演会 講演要旨集，56 (B-10)．

Kashiwada, E., K. Kuroda and G. Endo (1998) Aneuploidy induced by dimethylarsinic acid in mouse bone marrow cells, Mutat. Res., 413, 33-38.

McGregor, D.B., I. Edwards, C.G. Riach, P. Cattanach, R. Martin, A. Mitchell and W.J. Caspary (1988) Studies of an S9-based metabolic activation system used in the mouse lymphoma L5178Y cell mutation assay, Mutagenesis, 3, 485-490.

MHLW; Ministry of Health, Welfare, and Labor (2004) (http://www.mhlw.go.jp/topics/2004/07/tp0730-1.html)

Moore, M.M., K. Harrington-Brock and C.L. Doerr (1997) Relative genotoxic potency of arsenic and its methylated metabolites, Mutat. Res., 386, 279-290.

Noda, Y., T. Suzuki, A. Kohara, A. Hasegawa, T. Yotsuyanagi, M. Hayashi, T. Sofuni, K. Yamanaka and S. Okada (2002) In vivo genotoxicity evaluation of dimethylarsinic acid in MutaMouse, Mutat. Res., 513, 205-212.

Omori, T., M. Honma, M. Hayashi, Y. Honda and I. Yoshimura (2002) A new statistical method for evaluation of L5178Y*Tk*(+/−) mammalian cell mutation data using microwell method, Mutat. Res., 517, 199-208.

Preisler, V., W.J. Caspary, F. Hoppe, R. Hagen and H. Stopper (2000) Aflatoxin B1-induced mitotic recombination in L5178Y mouse lymphoma cells, Mutagenesis, 15, 91-97.

WHO; World Health Organization (2004) Guideline for Drinking-Water Quality, 3rd Edition Vol. 1. (http://www.who.int/water_sanitation_health/dwq/guidelines/en/index.html)