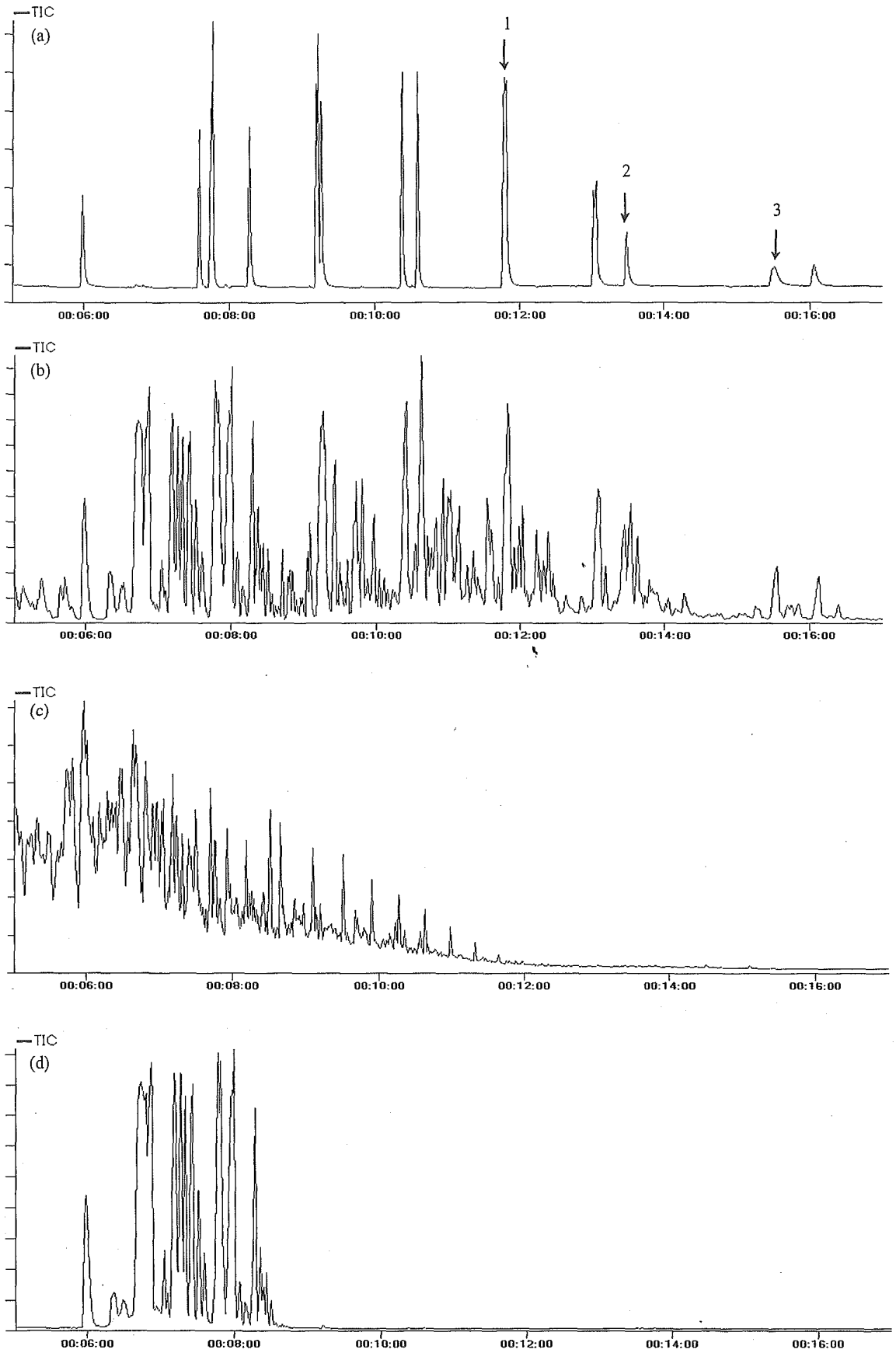

Fig. 1. Total Ion Chromatogram of Creosotes

(a) PAH standard solution (5 μg/ml), (b) sample No. 8, (c) sample No. 9, (d) sample code C. Peak 1: benz[*a*]anthracene, 2: benzo[*a*]pyrene, 3: dibenz[*a*,*h*]anthracene.

Table 6. PAHs Content in Creosote-Treated Wood Products

	Content (μg/g)					
	A	B	C	D	E	F
	Foundation	Railway Sleeper	Railway Sleeper	Railway Sleeper	Railway Sleeper	Stake
	New	New	New	Used	Used	New
Benz[*a*]anthracene	668	193	274	206	455	2
Chrysene	535	172	350	177	562	2
Benzo[*b*]fluoranthene	278	75	230	154	323	1
Benzo[*k*]fluoranthene	156	44	113	103	189	1
Benzo[*a*]pyrene	141	47	126	88	168	ND
Indeno[1,2,3-*cd*]pyrene	41	16	48	20	186	ND
Dibenz[*a*,*h*]anthracene	12	3	18	6	18	ND
Benzo[*ghi*]perylene	31	13	69	14	203	ND

ND means < 1.0 μg/g.

amount in all samples, but still exceeded 10 μg/g in No. 9 and in code C. This finding indicated that some wood preservatives, even those lacking creosote as a constituent, possibly contain high levels of PAHs, *i.e.*, those above the control amount. It is therefore necessary to be aware of the amount of PAHs, not only in cases involving creosote, but also in cases involving oil-type wood preservatives, in which creosote is not listed as a constituent. Sample code A was the only product tested here that conformed to the Japanese regulations.

Sample sections were taken approximately at 2 cm in depth from the surface of wood products manufactured in accord with the Japan agricultural standards (JAS) for the timber of the broadleaf tree. The samples were cut and treated with dichloromethane, and the amount of PAHs contained therein was determined (Table 6). In the commercially available samples A–E, amounts of PAH in excess of 3 μg/g were detected. These findings indicated that creosotes containing more than 10 μg/g each of BaA, BaP, and DBA had been used in the preservation of these samples. A small amount of PAHs per stake material was also detected. Macroscopic studies of sections taken 1 mm from the surface revealed a change in color, which indicated that the creosote had not penetrated to the center of the wood, and instead remained near the surface. Accordingly, almost all of the layers cut from the stake sample contained no creosote, and the total PAH content was low in the test solution prepared from this sample.

In conclusion, we improved upon our previous analytical method for determination of PAHs in creosotes and in creosote-treated woods. We adopted an evaporation-concentration step and changed the column temperature conditions in order to increase the sensitivity of testing and to reduce the amount of time needed to perform GC-MS. The result of a collaborative study indicated that the analytical method developed here appears to be sufficiently stable and can be used for the determination of low levels of BaA, BaP, and DBA. We found that these compounds in high concentrations, thus exceeding the allowed control value, were contained in some creosotes and railway sleepers.

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Monitoring of polycyclic aromatic hydrocarbons and water-extractable phenols in creosotes and creosote-treated woods made and procurable in Japan

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Abstract

The recycling of disused railway sleepers treated with wood preservatives such as creosote as exterior wood for use in gardens has recently become popular in Japan. Creosote contains high quantities of polycyclic aromatic hydrocarbons (PAHs), and can lead to skin irritation and disease. In this work we have determined the amount of PAHs and water-extractable phenols in creosote and creosote-treated wood products such as railway sleepers and stakes for agricultural use that are either made or are procurable in Japan. PAHs were extracted with dichloromethane and analyzed by gas chromatography–mass spectrometry. Among carcinogenic PAHs, benz(*a*)anthracene was detected in the highest concentration, varying between 228 and 6328 μg/g in creosotes. Benzo(*b*)fluoranthene, benzo(*k*)fluoranthene and benzo(*a*)-pyrene (BaP) were found in the range of 67–3541 μg/g. Almost all creosotes contained more than 50 μg/g of BaP, which is the upper limit level that is permitted in the European Union (EU). Creosote-impregnated wood products, such as brand-new or secondhand railway sleepers and foundations, contained large amounts of BaP (58–749 μg/g) and benz(*a*)anthracene (250–1282 μg/g). Concentrations of between 692 and 2489 μg/g of phenols were determined in the water extracts from creosotes, but the level was considerably less than the EU control value (3% by mass), and there was no correlation between the amount of water-extractable phenols and the amount of PAHs detected in each sample. The situation that consumers are free to use the creosotes containing a high concentration of carcinogens such as BaP may cause unacceptable damage to the health of persons handling these creosote products.

Keywords: Polycyclic aromatic hydrocarbons; Creosote; GC–MS; Phenols; Wood preservative

1. Introduction

Creosote is a mid-heavy distillate of coal tar with a boiling point of between 200 and 400 °C (Gevao and Jones, 1998). The annual production of creosote in Japan is about 70 million tons. The majority of this is used as raw material for carbon black, while much of the rest has been used as a wood preservative (market research of Journal of The Japan Aromatic Industry Association, Inc. and Japan Wood Preserving Association). Wood treated with creosote was formerly used for railway sleepers and poles for the transport of electricity. These items are now commonly used in the foundations

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of buildings, for building fences and as stakes for agricultural and fruit production, as well as in the manufacture of garden furniture and outdoor recreational facilities in parks.

One concern involved in the use of creosote is the long-term release into the environment of a wide variety of constituents that are present within creosote-treated wood (Bestari et al., 1998). Creosote can be toxic to animals, and direct contact with creosote can lead to skin irritation and disease (Agency for Toxic Substances and Disease Registry, 2002). The International Programme on Chemical Safety selected 33 polycyclic aromatic hydrocarbons (PAHs) for toxicological evaluation in Environmental Health Criteria (1998), and a variety of these PAHs are contained in high quantities (up to 85%) in creosote (Gevao and Jones, 1998). The US Environment Protection Agency (EPA, 1985) defined 16 unsubstituted PAHs as priority pollutants, which account for between 20% and 40% of the total weight of a typical creosote (Kohler et al., 2000). In the commission decision of European communities (2002), 12 PAHs were focused as the most important ones. Among the PAHs, benzo(*a*)pyrene (BaP) is one of the most thoroughly investigated and is classified in Group 2A of potential human carcinogens (IARC, 1983), so it was chosen as a marker for creosote treatment and is also taken as an indicator for the toxicity of creosote. Creosote was reported as being probably equally carcinogenic to humans (Group 2A) by the IARC (1987). The European Union (EU) has adopted limits to the marketing and use of creosote and creosote-treated wood according to a Council Directive in 1994. The Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE, 1999) related that the cancer risk from creosote is greater than previously thought, and exceeded the limits permissible under existing registration. Based on those concerns, a new Directive (2001/90/EC) was adopted by the European Council (Commission of the European Communities, 2001), and creosote that contains BaP at a concentration of higher than 0.005% (50 ppm) by mass and water-extractable phenols at a concentration of higher than 3% by mass may not be used in the treatment of wood and wood so-treated may not be place on the market.

The recycling of disused railway sleepers as exterior wood for use in gardens has recently become popular in Japan. Disused railway sleepers imported from China and other countries or obtained from domestic railroad companies have been sold at retail stores that deal in carpenter's or gardening tools. New exterior wood products including sleepers treated with creosote can also be obtained in the shops. Consequently, the opportunity to come into contact with creosote is increasing for the general public. Though these railway sleepers had been exposed to the weather for several decades, large concentrations of PAHs have been reported to exist as residues in this type of wood (Rotard and Mailahn, 1987; Kohler et al., 2000). Therefore, when the general public buys and handles such wood, which is even occasionally used for interior decoration, the numerous chemicals that are present as residues may impair their health. Each country in the EU has been restricting the use of creosote since 2003, but in Japan there is no control on the marketing and use of creosote and creosote-treated woods. To reduce the health risk from creosote in Switzerland, creosote with lowered BaP content has already been used (Kohler et al., 2000). In the present study, we determined the constituents (PAHs and water-extractable phenols) in creosote and creosote-treated wood (railway sleepers and stakes for agriculture) that are available on the market in preparation for regulation in Japan.

Creosote-treated wood has generally been extracted in a Soxhlet-apparatus (Rotard and Mailahn, 1987; Gevao and Jones, 1998; Kohler et al., 2000; Becker et al., 2001). Schwab et al. (1999) and Eriksson et al. (2001) reported that mechanical shaking and liquid extraction methods are also useful for analyzing petroleum hydrocarbons from soil, as well as Soxhlet extraction techniques. We examined whether soak extraction could be used as an alternative to Soxhlet extraction. A gas chromatography (GC) system equipped with a capillary column is generally used for the determination of PAHs in various fields, including wood products, water, air and the petro-chemical industry. Furthermore, mass selective-ion-monitoring is useful for detecting each chemical selectively and provides sufficient separation for quantification of the PAHs in a complex such as creosote. (Rotard and Mailahn, 1987; EPA, 1993; Woolgar and Jones, 1999; Kohler et al., 2000; Becker et al., 2001; Eriksson et al., 2001; Shu et al., 2003; Ou et al., 2004). Bestari et al. (1998) investigated the PAH content in wood products and the leaching behavior of creosote-treated wood by high-performance liquid chromatography (HPLC). Anklam et al. (1997) used an HPLC system equipped with a fluorescence detector for the determination of the BaP content in creosote. HPLC determination is sensitive to PAHs, but the identification of individual PAHs by comparison of their retention time is less accurate compared with GC. Therefore, in this study, we used GC–mass spectrometry (GC–MS) for the determination of PAHs in creosotes.

2. Experimental section

2.1. Samples

Four commercially-available creosotes (sample A–D) and five creosotes (E–I) provided by the Japan Aromatic Industry Association, Inc. (JAIA) were investigated. Three oil-based wood preservative paints (NA, NB

Table 1
List of wood products tested

No.	Use	Type of wood			
1	Foundation	Japanese hemlock	Creosote treated	New	Made in Japan[a]
2	Railway sleeper	Kempas	Creosote treated	New	Made in Japan[a]
3	Railway sleeper	Kempas	Creosote treated	New	Made in Japan[a]
4	Railway sleeper	Pine tree	Creosote treated	Used	Imported from China
5	Railway sleeper	Unknown	Creosote treated	Used	Imported from China
6	Railway sleeper	Pine tree	Non-treated	New	Made in Japan
7	Stake	Japanese cedar	Creosote treated	New	Made in Japan
8	Stake	Japanese cedar	Non-treated	New	Made in Japan

[a] Supplied by the Japan Wood Preservers Industry Association (JWPIA).

and NC) that did not contain creosote were used for comparison. Used or new railway sleepers, foundation and wooden stakes were obtained and supplied by the Japan Wood Preservers Industry Association (JWPIA). The wood products tested were listed in Table 1.

2.2. Chemicals

An EPA polycyclic aromatic hydrocarbon (PAH) mixture containing acenaphthene, acenaphthylene, anthracene, benz(*a*)anthracene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*ghi*)perylene, benzo(*a*)pyrene (BaP), chrysene, dibenz(*a,h*)anthracene, fluoranthene, fluorene, indeno(1,2,3-*cd*)pyrene, naphthalene, phenanthrene and pyrene at a concentration of 2000 μg/ml in dichloromethane–benzene (1:1) (Supelco, USA) was used as a standard for the PAHs. The PAH standard mixture was diluted with dichloromethane to reach a final concentration of 10 μg/ml. Acenaphthene-d10, chrysene-d12 and phenanthrene-d10 (Supelco, USA or Wako Pure Chemical Industries, Ltd., Japan) were obtained as an internal standard for PAH determination. Each internal standard was dissolved in dichloromethane at concentrations of 5–20 μg/ml. All of the other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Japan. A standard stock solution of phenol was prepared by dissolving phenol in water to a concentration of 100 μg/ml. A 4-aminoantipyrin reagent was prepared by dissolving 1.36 g of the reagent in 100 ml of water. A boric acid (H_3BO_3) buffer solution was used that consisted of a mixture of a 1 mol/l sodium hydroxide (NaOH) solution and a 3 mol/l H_3BO_3 solution at a ratio of 9:10. A potassium ferricyanide ($K_3Fe(CN)_6$) reagent was prepared by dissolving 8.6 g of $K_3Fe(CN)_6$ and 1.8 ml of an ammonium solution in water to a volume of 1000 ml.

2.3. Instrument

An ultraviolet spectrometer (Shimadzu UV-160A) was used for the determination of the phenols. A gas chromatograph (GC) (Hewlett–Packard GC5890 Series II Plus) connected to a JEOL JMS-AM II 20 mass spectrometer (MS) (GC–MS) was used for analysis of the individual PAHs. System control and data processing of the GC–MS were performed using the JEOL Automass II program (JEOL Datum). The column was a PTE™-5 fused silica capillary column (30 m length and 0.25 mm i.d. coated with a 0.2 m thickness of 5% diphenyl–95% dimethylpolysiloxane film, Supelco). Sep-Pak Plus silica-cartridges (Part No. WAT020520) for solid extraction were obtained from Waters Co. (Massachusetts, USA).

2.4. Analysis of PAH

2.4.1. Extraction

For creosote oils, the weight (g) of a 0.1 ml sample was measured in advance. The Sep-Pak Plus silica-cartridge was conditioned by rinsing with 10 ml of dichloromethane. 0.1 ml of the sample was loaded onto the cartridge, and then eluted with 10 ml of dichloromethane into the volumetric flask. Dichloromethane was added into the flask to a final volume of 20 ml.

For extraction from wood products, a section approximately 3 cm in depth from the surface of the samples was removed and cut into small pieces about 2–3 mm side and 3 cm long. In other analysis of stakes, only the surface (approximately 3 mm in depth) was shaved off. A portion of the 1.0 g fragment was weighed into a 15 ml screw-capped glass tube. After adding 10 ml of dichloromethane, the tube was incubated for 24 h at 37 °C. The extract was filtrated and loaded onto a Sep-Pak Plus silica-cartridge that was conditioning described above. The wood sample and tube were washed with a small amount of dichloromethane and these washings were also added into the cartridge. The cartridge was further eluted with 10 ml of dichloromethane and the extraction solution was adjusted to a total of 25 ml.

2.4.2. GC–MS determination

The extraction solution was diluted with dichloromethane, and a series of diluted sample solution (1×,

Table 3
Effect of extraction condition on the recovery of PAH from creosote-treated wood samples

Chemical	Found (μg/g)			
	Shake (rt, 1 h)	Stand (rt, 24 h)	Stand (37 °C, 24 h)	Soxhlet-extraction (50 °C, 24 h)
Naphthalene	83	176	295	383
Acenaphthylene	85	114	173	158
Acenaphthene	2811	4388	4854	5740
Fluorene	3305	4178	4385	5090
Phenanthrene	11841	17020	17578	12620
Anthracene	4249	5150	4728	4790
Fluoranthene	4536	5900	6406	5590
Pyrene	3577	4360	4494	4740
Benz(*a*)anthracene	1013	1104	1019	989
Chrysene	987	1067	976	939
Benzo(*b*)fluoranthene	327	471	558	377
Benzo(*k*)fluoranthene	238	286	382	309
Benzo(*a*)pyrene	206	267	380	280

rt = room temperature.
Creosote-treated wood was cut in small pieces and 1.0 g was weighed into a tube. Dichloromethane 10 ml was added and the sample was extracted using each of the conditions listed. In the Soxhlet-extraction, 5.0 g of the sample was extracted with 100 ml of dichloromethane at 50 °C for 24 h. The eluate was taken by filtration and 1 ml of the eluate was loaded on a Sep-Pak silica cartridge and eluted with 10 ml dichloromethane. The eluate was adjusted to an appropriate volume with dichloromethane and injected into the GC–MS. The concentration of PAH in the sample was measured.

Table 4
Content of PAHs in wood products

Chemical	Amount (μg/g)							
	1	2	3	4	5	6	7	8
Naphthalene	374	1017	1064	439	338	7	303	6
Acenaphthylene	212	290	245	197	232	4	187	4
Acenaphthene	2000	4251	8043	2355	449	ND	266	4
Fluorene	1764	3131	2754	3207	390	ND	101	ND
Phenanthrene	6040	6069	1270	10619	7837	ND	105	ND
Anthracene	1831	1026	915	2573	1124	4	33	5
Fluoranthene	2447	1144	843	6013	7966	ND	24	ND
Pyrene	1411	813	731	3702	7278	ND	14	ND
Benz(*a*)anthracene	787	250	336	1282	1058	ND	ND	ND
Chrysene	715	226	401	875	1094	ND	ND	ND
Benzo(*b*)fluoranthene	256	88	192	194	973	ND	ND	ND
Benzo(*k*)fluoranthene	157	62	94	154	601	ND	ND	ND
Benzo(*a*)pyrene	134	58	130	125	749	ND	ND	ND
Indeno(1,2,3-*cd*)pyrene	14	ND	58	13	323	ND	ND	ND
Dibenz(*a,h*)anthracene	ND	ND	7	ND	35	ND	ND	ND
Benzo(*ghi*)perylene	8	ND	54	9	288	ND	ND	ND

ND means < 4 μg/g.

suggested that the PAHs detected were derived from the treatment with creosote. We guess that the difference in the PAH content in the samples was caused by the difference in the manufacturing method of creosote and the impregnation amount of creosote into the woods.

Kohler et al. (2000) reported that naphthalene and acenaphthene in the top layer of railroad ties had leached into the environment with aging, and carcinogenic compounds such as benz(*a,h*)anthracene and BaP etc remained at the same level in the products over an extended time, so the relative concentrations of these chemicals were significantly higher in the top layer than in the inner layers. It suggested that the determination of the PAH content in the outer layers is important for assessing the risk to human health following skin contact with creosote-treated products. The creosote con

tent in the railway sleepers and foundations those are creosote-impregnated wood products is not significantly different between the outer layers and the center layers. In contrast, the amount of PAHs in a stake sample (No. 7) is small. Macroscopic studies of sections taken 1–2 mm from the surface showed a change in color, so creosote does not penetrate into the center of the wood but remains near to the surface. Accordingly, almost all of a layer cut approximately 3 cm in depth from the surface of a stake does not contain any creosote, and the total creosote content was low when a test solution was prepared. In a preliminary experiment, the dissipation pattern of PAHs in an extract prepared from a section taken 2 mm in depth from the surface of a stake was comparable to that observed in the extract from a railway sleeper or from the liquid creosote used in this study. It is believed that the creosote used to treat the stake was not a special grade product with decreased four- to six-ring PAH content. No significant occurrences of skin cancer have been reported in workers in creosote-impregnation plants or in consumers, but CSTEE (1999) warned of the cancer risk from creosote containing 50 μg/g BaP and from wood treated with such creosote.

3.3. Water-extractable phenols in creosote

The difference of phenols amount extracted from creosote by increased shaking time from 10 min to 2 h was small. In this study, a creosote sample was shaken with water for 30 min at room temperature, and the water layer was obtained by centrifuging. Table 5 presents the content of water-extractable phenols in creosote and wood preservative paints. Typical phenol concentrations of between 692 and 2489 μg/g were found in creosotes. In contrast, lower concentrations of phenols were found in non-creosote-type wood preservatives. There was no correlation between the amount of water-extractable phenols and the amount of PAHs in each sample. The levels of phenols determined in these creosotes were considerably lower than the maximum levels permitted in the EU (3% by mass) and were similar to the data (between 0.06% and 0.77%) reported by Kohler et al. (2000). Creosotes that are commercially available in Japan receive an alkaline treatment after distillation (personal communication from a manufacturer), so the content of phenols is believed to be low.

Table 5
Content of water-extractable phenols in creosotes and non-creosote type wood preservatives

Sample		Water-extractable phenols (μg/g)
Creosote	A	1093
	B	1415
	C	692
	D	782
	E	2121
	F	2155
	G	1294
	H	2359
	I	2489
Wood preservative (non creosote)	NA	378
	NB	31
	NC	ND

The sample (0.1 g) was shaken with 5 ml water for 30 min and the water layer was obtained by centrifuging at 3000 rpm for 5 min. The content of water-extractable phenols was determined by the 4-aminoantipyrin method.

3.4. Water-extractable phenols in creosote-treated wood

There are a few reports on the investigation of water-extractable phenols in creosote-treated wood samples. In our preliminary examination, the detected phenol content increased with increasing extraction time and temperature. This means that migration of water into the sample for the extraction of phenols trapped inside the wood took a long time, and the quantity of water-extractable phenols detected varied with the extraction temperature and the incubation period.

Samples were extracted by autoclaving or incubation for 24 h at 37 °C. For all samples, the values obtained with the autoclave extraction process were approximately two to three times as large as those determined after the 24 h incubation process (Table 6). The phenol content was almost the same for railway sleepers and stakes that were treated or not treated with creosote under both conditions. It was thought that almost all of the water-extractable phenols determined in this study did not originate from the creosote-treatment but from phenol-structural compounds that were primarily contained in the wood or by the generation of oxidized compounds due to hydrolysis during the extraction process. The amount of phenols in the

Table 6
Content of water-extractable phenols in creosote-treated wood products

Sample	Creosote treatment	Water-extractable phenols (μg/g)	
		Autoclave (121 °C, 10 min)	Incubation (37 °C, 24 h)
1	Yes	706	319
2	Yes	363	181
3	Yes	392	176
4	Yes	533	50
5	Yes	360	74
6	No	425	113
7	Yes	324	158
8	No	529	193

creosote is also relatively low (Table 5), and the release of creosote from wood samples into water is minimal (Becker et al., 2001). Therefore, it is not important to measure water-extractable phenols in commercially-available wood samples.

4. Conclusion

This study demonstrated that varying amounts of PAHs and water-extractable phenols are present in creosote and creosote-treated wood products such as railway sleepers and stakes that are sold for agricultural purposes. Among carcinogenic PAHs, benz(*a*)anthracene was detected in the highest concentration, varying between 228 and 6328 μg/g in creosotes. Benzo(*b*)fluoranthene, benzo(*k*)fluoranthene and BaP were found in the range of 67–3541 μg/g. Almost all creosotes contained more than 50 μg/g of BaP, which is the upper limit level that is permitted in the EU standard. Creosote-impregnated wood products, such as brand-new or secondhand railway sleepers and foundations, also contained significant amounts of BaP (58–749 μg/g) and benz(*a*)anthracene (250–1282 μg/g). The concentration of phenols was low in creosotes and creosote-treated wood, and was not related to PAHs content. The effects of the water-extractable phenols on health might to be negligible. In Japan, creosotes containing a high concentration of BaP have been sold, and consumers are free to use them for wood preservation. This situation may cause impermissible health damage to persons handling creosotes and creosote-treated wood products, and the government has scheduled a restriction of the use of creosotes containing elevated amounts of carcinogenic PAHs.

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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 C Ltd. (Noda, Chiba, Japan), which were prepared from th liver of phenobarbital- and 5,6-benzoflavone-treated SD rat We prepared a 10 ml S9 mix with 4 ml S9 fraction and 2 n each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP ar 150 mM KCl.

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

2.2. Comet assay

After treating the cells for 4 h with AA or GA, we pr pared slides for alkaline COM assay as previously reporte [15]. Briefly, the cells were suspended in 0.5% agarose-LG (Nakalai Tesque Inc., Kyoto, Japan), quickly layered on a slic (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 solutic (pH 13) for 1 h, electrophoresed it for 15 min after the unwin ing treatment, fixed the cells with 70% ethanol, and staine them with SYBER green (Molecular Probes, Eugene, OI according to the manufacturer's recommendation. We observe the cells by an Olympus model BX50 fluorescence microscop At least 50 cells were captured by CCD camera, and the t length of the comet image was measured. We statistically an lyzed the difference between the non-treated and treated plat with the Dunnett's test after one-way ANOVA [16].

2.3. Micronuclei test

Forty-eight hours after treatment, we prepared the MN te samples as previously reported [17]. Briefly, approximately 1 cells suspended in hypotonic KCl solution were incubated f 10 min at room temperature, fixed twice with ice-cold glaci acetic acid in methanol (1:3), and resuspended in methan containing 1% acetic acid. We placed a drop of the suspe sion on a clean glass slide and allowed it to air-dry. V stained the cells with 40 μg/ml acridine orange solution a immediately observed them by Olympus model BX50 flu rescence microscope. At least, 1000 intact interphase cells f each treatment were examined, and the cells containing M were scored. The MN frequencies between non-treated a treated cells were statistically analyzed by Fisher's exact te The concentration–response relationship was evaluated by t Cochran–Armitage trend test [18].

2.4. TK gene mutation assay

The TK6 cell cultures were maintained for 3 days af treatment to permit expression of the *TK* deficient phenoty To isolate the *TK* deficient mutants, we seeded cells from ea

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-

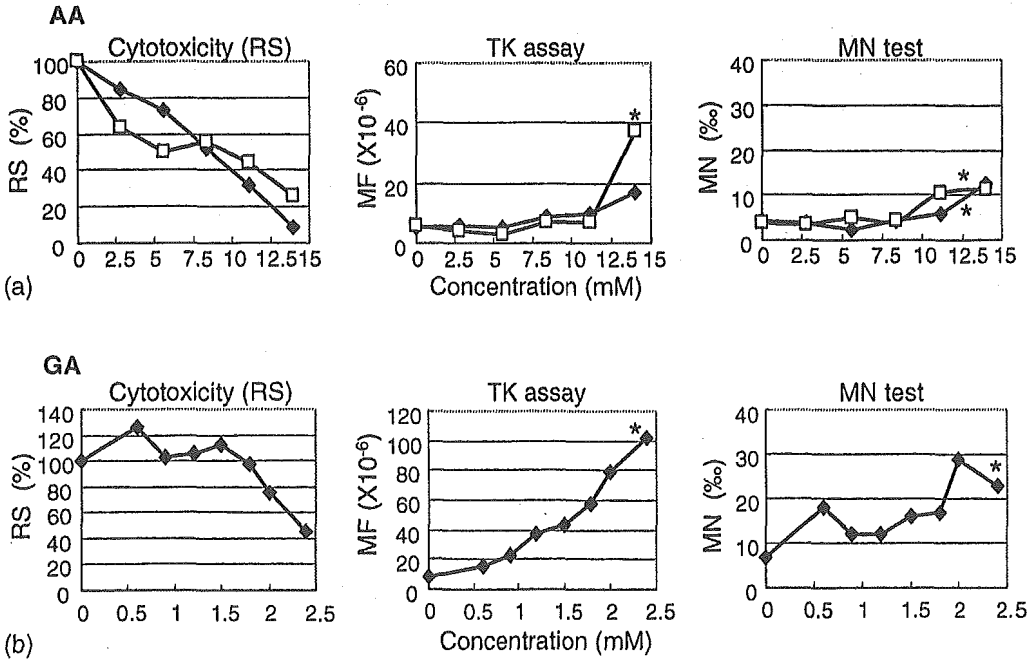

Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, 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-mutar

Treatment	Cytotoxic and mutational response			LOH analysis at TK gene			
	RS (%)	MF ($\times 10^{-6}$)	% SG	No.	None LOH	Hemi-LOH	Homo-LC
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 spontaneous occurring *TK* mutants described previously [16]. Tl fraction of hemi-LOH in AA-induced mutants, in whic 50% each of NG and SG mutants exhibited hemi-LOI was higher than in spontaneous mutants, indicating th AA-induced primarily deletions. GA, on the other han induced primarily NG mutants, and most (93%) of the were the non-LOH type, which is presumably generate by point and other small intragenic mutations. Amor 16 GA-induced SG mutants, the percentages that we hemi-LOH (38%) and homo-LOH (62%) were simil to those observed in spontaneous SG mutants. Fig. shows the mutation spectra of *TK* mutants found amor treated and untreated TK6 cells. GA and ethyl metha sulfonate, an alkylating agent, produce similar spectr as do AA and X-radiation.

Fig. 4 shows the distribution of LOH in AA-induce ($n = 37$), GA-induced ($n = 17$) and spontaneous ($n = 2$ LOH mutants. Because the majority of GA-induce mutants were the non-LOH type, we were able to ma only 17 GA-induced LOH mutants. As a particular cha acteristic of AA-induced LOH mutants, we frequent observed small deletions limited to the *TK* locus. Tl

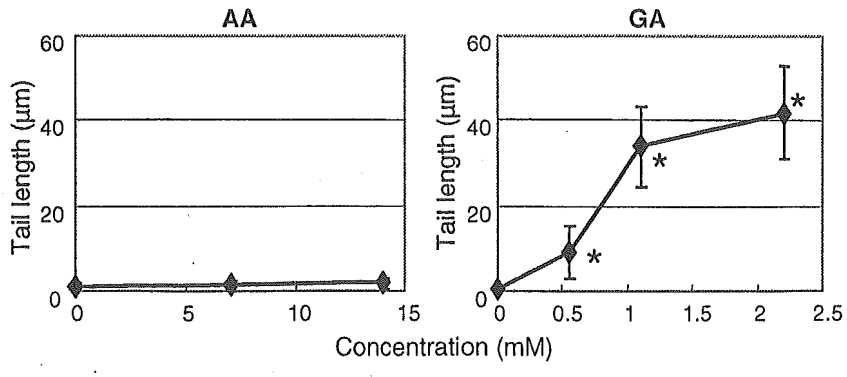

Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (*) statistically significant in t Dunnett's tests ($P < 0.05$).

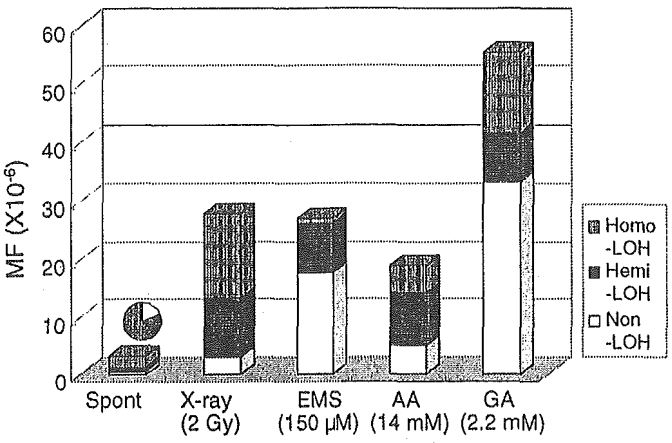

Fig. 3. Frequency and spectra of TK mutations in spontaneous and X-ray-induced (2 Gy), EMS-induced (150 μM, 4 h), AA-induced (14 mM, 4 h) and GA-induced (2.2 mM, 4 h) TK mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of normally growing (NG) and slowly 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

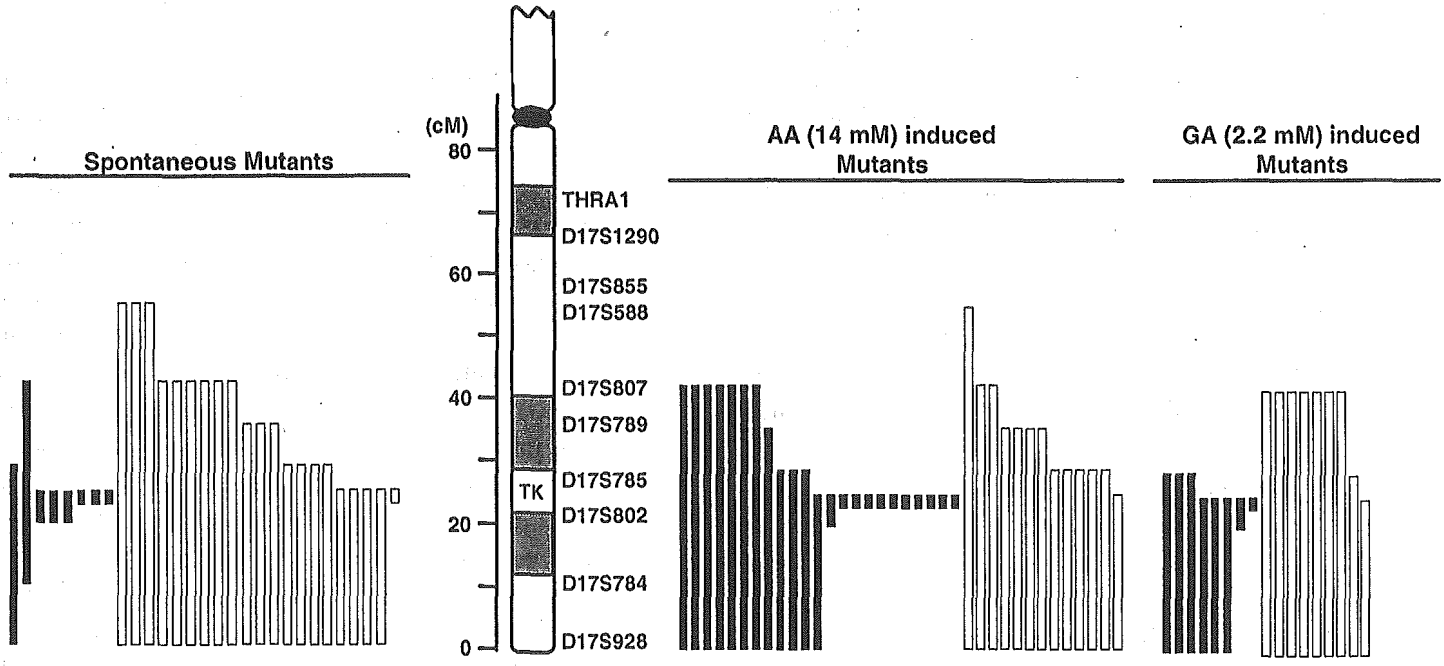

Fig. 4. The extent of LOH in spontaneous, AA-induced and GA-induced LOH mutants from TK6 cells. We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human TK locus maps to 17q23.2. Open and closed bars represent homo-LOH and hemi-LOH, respectively. The length of 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].

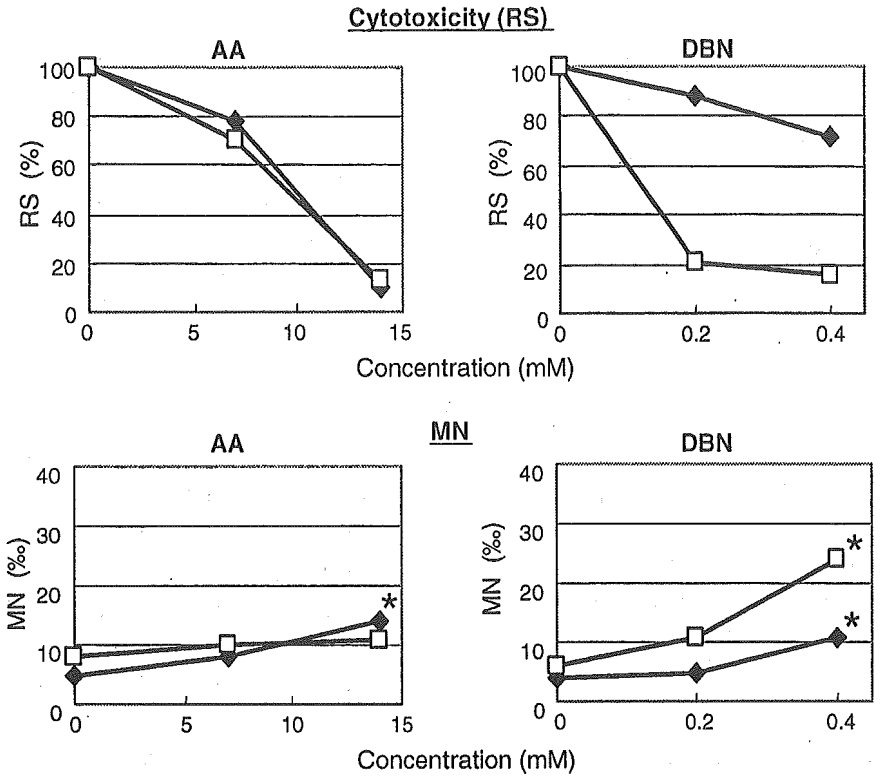

Fig. 5. Cytotoxicity (RS) and MN induction in TK6 cells treated with AA or DBN for 4 h in the presence (open symbol) or absence (closed symbc of rat liver S9. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

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 mutatic assay [29]. In contrast to AA, GA is a mutagen, inducir primarily point mutations.

AA is known to be metabolized to GA by CYP2E [9]. GA, an epoxide, forms adducts directly wi DNA and protein, causing cytotoxicity and genotoxi ity. GA forms mainly *N*7-(2-carbamoyl-2-hydroxyethy guanine and *N*3-(2-carbamoyl-2-hydroxyethyl) adenir and reacts with hemoglobin and cytoskeletal protei [31–33]. Rat S9, however, did not affect AA cytotoxici or genotoxicity, although it did enhance the cytotoxici and genotoxicity of DBN, which is also metabolized b CYP2E1. This suggests that rat S9 does not work f activating AA. AA and GA are detoxified through gl tathione conjugation, and GA is also detoxified by epo hydrolase (EH), which catalyzes the hydrolysis of GA dihidroxy propionamide [34,35]. Other in vitro studi also failed to demonstrate the enhancement of AA gen toxicity by rat S9 [36,37]. Our results do not mean th AA is always detoxified rather than activated becau DNA adducts are found in mice and rats given oral A and the genotoxicity of AA is consistently observed in vivo studies [8,31,36,37]. Recently, Manjanatha et a demonstrated in transgenic Big Blue™ mice that AA well as GA induces endogenous *Hprt* and transgenic c mutation at same level, and both chemicals cause pr dominantly base substitutions and frameshift mutatior

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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In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals

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Abstract

Genotoxicity is one of the important endpoints for risk assessment of environmental chemicals. Many short-term assays to evaluate genotoxicity have been developed and some of them are being used routinely. Although these assays can generally be completed within a short period, their throughput is not sufficient to assess the huge number of chemicals, which exist in our living environment without information on their safety. We have evaluated three commercially available in silico systems, i.e., DEREK, MultiCASE, and ADMEWorks, to assess chemical genotoxicity. We applied these systems to the 703 chemicals that had been evaluated by the Salmonella/microsome assay from CGX database published by Kirkland et al. [1]. We also applied these systems to the 206 existing chemicals in Japan that were recently evaluated using the Salmonella/microsome assay under GLP compliance (ECJ database). Sensitivity (the proportion of the positive in Salmonella/microsome assay correctly identified by the in silico system), specificity (the proportion of the negative in Salmonella/microsome assay correctly identified) and concordance (the proportion of correct identifications of the positive and the negative in Salmonella/microsome assay) were increased when we combined the three in silico systems to make a final decision in mutagenicity, and accordingly we concluded that in silico evaluation could be optimized by combining the evaluations from different systems. We also investigated whether there was any correlation between the Salmonella/microsome assay result and the molecular weight of the chemicals: high molecular weight (>3000) chemicals tended to give negative results. We propose a decision tree to assess chemical genotoxicity using a combination of the three in silico systems after pre-selection according to their molecular weight.

Keywords: In silico; (Quantitative) structure-activity relationship; (Q)SAR; Chemical genotoxicity; Decision tree

1. Introduction

It is said that more than 20,000 chemicals are in use in Japan. Among them, only approximately 10% are thought to have been assessed for human hazard based on data from in vitro and in vivo bioassays. According to the "Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc." [2], the Salmonella/microsome (Ames) assay, in vitro chromosomal aberration assay (or alternatively mouse lymphoma TK assay), and 28-day repeat dose toxicity test in rodents are obligatory to notify new chemicals for production/import at a level of more than 10 t per year.

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To screen the remaining 18,000 chemicals for human hazard by application of this three-test battery is not realistic from the time and economical point of view. We need a much higher-throughput system to assess these chemicals, at least for prioritization of those chemicals that should be submitted to biological testing. To assess human hazard for regulatory purposes, in silico systems are now beginning to be used [3]. Here, we evaluated three commercially available in silico (quantitative) structure-activity relationship ((Q)SAR) systems and tried to construct a decision tree for prioritization of which chemicals need in vitro and/or in vivo testing. Also, within the drug discovery process, integrated computational analysis has been proposed to be incorporated as a toxicity prediction tool [4].

Kirkland et al. [1] published a database (CGX database, see http://www.lhasalimited.org/cgx) for nearly 1000 carcinogens and non-carcinogens with results of representative in vitro genotoxicity assays, i.e., Salmonella/microsome assay (Ames), mouse lymphoma TK assay using L5178Y cells (MLA), and in vitro chromosomal aberration assay or in vitro micronucleus assay (CA/MN). We used 703 chemicals that had been assessed in the Ames assay for evaluation of the three in silico systems, i.e., DEREK, MultiCASE (MCase), and ADMEWorks (AWorks). We also used a database (the ECJ database) that we constructed from chemicals existing in Japan that had recently been assessed in the Ames assay, in vitro chromosomal aberration assay, and 28 day repeat dose rodent toxicity test and/or reproductive and developmental toxicity test for their safety evaluation under GLP compliance. The ECJ database consisted of 206 chemicals but only 26 chemicals were positive by the Ames assay. Initially we evaluated both sensitivity and specificity of these three systems using the ECJ database of 206 chemicals [5].

We selected these three in silico systems because of their different modes of analysis. DEREK is a rule-based system [6], MCase [7] is a database/substructure based system, and AWorks is a QSAR. We applied these systems individually to assess gene-mutation induction on the 703 and 206 chemical sets described above and evaluated their sensitivity, specificity, concordance, and applicability (how many chemicals could be assessed), independently.

It is known that high molecular weight polymers tend not to induce gene mutation and chromosomal aberrations mainly because they cannot enter the target cells to react with DNA, or other bio-molecules necessary for genetic stability. We analyzed 194 Ames positive chemicals (confidential source) for the effect of molecular weight.

2. Materials and methods

2.1. Data sources for chemicals assessed

Of about 1000 chemicals, 703 that had been assessed the Ames test were chosen from the CGX database publishe by Kirkland et al. [1]. All chemical structures were re-draw using Chemdraw Ultra (Cambridge Soft Corporation, US/ and converted to MOL files before application to each sy tem. We also used the database of 206 chemicals evaluated the MHLW project "Safety Examination of Existing Chen icals and Safety Programmes in Japan" (ECJ database). Tl test summary for each of these chemicals can be seen http://wwwdb.mhlw.go.jp/ginc/html/db1.html. In addition, v collected 194 Ames positive chemicals from a confidenti source and investigated the relationship between gene mut tion induction and molecular weight, with identification of ar active side chain that might have contributed to the positi result in the Ames assay.

2.2. In silico systems used and definition of positive and negative responses

We used DEREK (Lhasa Ltd., UK) version 8.0.1.When th system gave an evaluation as "certain", "probable" or "plaus ble" we considered this as "positive", and when the system ga "equivocal", "doubted", "improbable", "impossible", or "r alert" we considered this as "negative". We used MCase (Mu ticase Co. Ltd.) version mc4pc. When the system gave "activ or "marginal" we considered this as "positive", and when th system gave "Inactive" we considered this as "negative". I the case of AWorks (Fujitsu Kitakyushu, Co. Ltd., version 2.0 we considered as "positive" when system evaluation was "pos tive", and considered as "negative" when the system evaluatic was "negative". We excluded chemicals from further analys when DEREK or AWorks gave no answer, or the evaluatic was "inconclusive" by MCase.

2.3. Definition of sensitivity, specificity, concordance, and applicability

We calculated sensitivity, specificity, concordance, ar applicability as follows:

$$\text{sensitivity} = \frac{N_{A+S+}}{N_{A+}} \times 100, \qquad \text{specificity} = \frac{N_{A-S-}}{N_{A-}} \times 10$$

$$\text{concordance} = \frac{N_{A+S+} + N_{A-S-}}{N_{\text{eval}}} \times 100,$$

$$\text{applicability} = \frac{N_{\text{eval}}}{N_{\text{all}}} \times 100$$

where N_{A+} is number of chemicals revealing positive in Am assay; N_{A-} is number of chemicals negative in Ames assa N_{A+S+} is number of chemicals revealing positive by both Am assay and in silico evaluation; N_{A-S-} is number of chemica negative in both Ames assay and in silico evaluation; N_{eval}