Introduction

Toluene-diisocyanate (TDI) is an important industrial chemical used in the production of polyurethane foams, elastomers, paints and coatings. TDI used in industry is an isomeric mixture of 2,6-TDI and 2,4-TDI in ratios of 20:80 or 35:65 [World Health Organisation (WHO) *1987*]. Diisocyanates are very reactive compounds, which covalently bind to endogenous macromolecules such as carboxyl groups and amino groups. Thus, skin irritation (Calas et al. *1977*), conjunctivitis (Luckenbach and Kieler *1980*), upper respiratory impairments and allergic asthma (Banks et al. *1986*; Baur et al. *1994*; Hagmar et al. *1987*) are well known as occupational health problems for workers using TDI. An obstructive pattern of pulmonary dysfunction was suggested to be shown in workers exposed to these chemicals (Omae et al. *1992*). The threshold limit value (TLV) of 2,4-TDI is 0.005 ppm [American Conference of Governmental Industrial Hygienists (ACGIH) *2003*]. The occupational exposure limit of TDI (the sum of 2,4-TDI and 2,6-TDI) is also recommended to be 0.005 ppm [Japanese Society for Occupational Health (JSOH) *1992*].

During the last decade, several methods were developed for the determination of the urinary metabolite (toluene-diamine, TDA) of TDI (Lind et al. *1997*; Maitre et al. *1993*; Morita et al. *2003*; Sakai et al. *2002*; Sandstrom et al. *1989*). The biological exposure index (BEI) of TDA has not been adopted by the ACGIH (ACGIH *2003*). The limited application of those methods to actual workers might be because the methods reported, so far, were of time-consuming hydrolysis (overnight) and needed a deuterated substance as the internal standard, which was expensive and not easily obtained commercially. In many of those methods, more than 1 ml (largest 3 ml) of urine was also needed for the analysis, and the hydrolyzed TDA was extracted with toluene, which was relatively less effective than other solvent (such as dichloromethane, diethyl ether, ethyl acetate, and so on) for TDA extraction (Sakai et al. *2002*).

In this study, we developed a sensitive and practicable GC-MS method for determining TDA after short-time hydrolysis of urine and applied the method to the biological monitoring of workers exposed to TDI.

Materials and methods

Subjects

Urine samples were collected from 18 male workers exposed to TDI in two factories and from 20 control subjects with no history of occupational exposure to TDI. TDI workers were engaged in the urethane painting of musical instruments. They were spraying urethane paints on musical instruments such as piano and guitar. TDI used in the factories was a mixture of two isomers, 2,4-TDA and 2,6-TDI, in the ratio 80:20. Urine was collected before and after the work shift (pre-shift and post-shift urine), and the samples were kept frozen at –20°C until required for analysis. All subjects gave informed written consent to participate in the study and agreed with the procedures. The medical ethics committee of Tokyo Rosai Hospital approved the study procedures, which conformed to the principals outlined in the Declaration of Helsinki.

Chemicals

2,6-TDA, 2,4-TDA, 3,4-TDA, and heptafluorobutyric anhydride (HFBA) were obtained from Tokyo Kasei (Japan). Sulfuric acid and dichloromethane (DCM) were purchased from Wako Pure Chemicals (Japan). The water used in the present study was either distilled water or water purified with a Millipore Milli-Q system (Nippon Millipore, Japan).

Sample preparation

The standard conditions used for the sample preparation in the present study were as follows: 0.1 ml of urine was diluted with 0.9 ml of water and then hydrolyzed by being boiled for 1.5 h with 0.1 ml of concentrated sulfuric acid in a total volume of 1.1 ml (11-fold dilution and a final concentration of 1.8 mol/l sulfuric acid). After hydrolysis, the urine samples were cooled and 0.7 ml of 8 mol/l sodium hydroxide solution was added. The alkalinized hydrolysate was then extracted with 2 ml of DCM for 20 min. After centrifugation at 3,000 rpm for 5 min, the organic layer was transferred to another tube. Fifty microliters of internal standard (200 ppb 3,4-TDA) and 50 μl of HFBA were added to each tube. The tubes were capped and shaken for 30 s. Derivatization with HFBA was carried out at 55°C for 1 h. After cooling, the DCM layer was dried to approximately 20–30 μl under a nitrogen steam. The residue was dissolved in 200 μl of toluene and used for GC-MS analysis.

In order to assess the standard method (the above-mentioned 11-fold dilution method), a 1.1-fold dilution was also examined in a total volume of 1.1 ml (1 ml of urine and 0.1 ml of concentrated sulfuric acid) and in a final concentration of 1.8 mol/l sulfuric acid. Hydrolysis was also carried out for 16 h for the comparison of hydrolysis yield to 1.5 h hydrolysis.

Analysis

Of the sample solution, 1 μl was injected into the GC-MS (Model GC-17A and QP5050A, Shimadzu, Japan) in splitless mode. The capillary column used was a DB-1 (30 m×0.25 mm, 0.25 μm film thickness; J&W Scientific, USA). Injection temperature was set at 280°C, and the column temperature was programmed from 100°C to 280°C at 20°C/min. Helium gas was used as the carrier, at a constant flow (1.3 ml/min). The samples were injected by splitless mode with a sampling time of 1 min. The GC-MS interface temperature was 280°C. The pressure in the ion source, in the case of NCI with iso-butane, was kept at ca. 1.2×10^{-3} Pa, and the detector gain was 1.25 kV. Under our condition as the routine, contamination of the ion source is not so fast. We can use the ion source more than 200 times without washings. The column can be used for more than 1,000 analyses. The TDA derivative was detected with the ion, m/z=494, in negative mode. Urinary creatinine concentrations were determined by Jaffe's method with a creatinine determination kit (Wako Pure Chemical, Japan: based on a colorimetric method). The specific gravity of urine was determined by protein refractometer (D type, Erma, Japan).

Determination of personal exposure levels of TDIs

Personal exposure levels were determined by the method of the Occupational Safety and Health Administration (OSHA; no. 42) (OSHA *1989*). Air samples were collected by use of a

personal sampling pump (GilAir, Gilian, USA) with the sampling device in line. The sampling device was a three-piece styrene cassette (225-3-01, SKC, USA) containing a glass-fiber filter (225–9002, SKC, USA) coated with 0.4 mg of 1-(2-pyridyl) piperazine and backup pad. Sampling was carried out for 4–6 h at a flow rate of 1 l/min. Samples were extracted with 2 ml of the mixture of acetonitrile/dimethyl sulfoxide (90:10) for 1 h, and analyzed by a high performance liquid chromatograph (HPLC; Gilson, France) with an ODS column (Symmetry C18, 3.9 mm×150 mm×5 μm, Waters, USA). TDI derivatives were detected by UV/VIS detector at 254 nm (119UV/VIS, Gilson, France). The mobile phase was the mixture of acetonitrile/10 mmol/l ammonium acetate (30:70) at flow rate of 1 ml/min.

Results

Figure 1 shows the chromatogram of three isomers of standard TDA and TDA isomers in urine from a TDI worker and a control subject. Three kinds of standard TDAs were eluted in the order of 3,4-, 2–6, and 2,4-TDA (Fig. 1c). 2–6-TDA, and 2,4-TDA were not detected in control urine (n=20), and 3, 4-TDA (internal standard) was detected neither in the hydrolyzed urine of workers nor in that of control subjects. The calibration curves of 2,6-TDA and 2,4-TDA were linear up to 400 μg/l. The detection limit of the TDAs was 0.1 μg/l. When 100 μg/l of individual TDA standard was added to ten control urine samples, the mean recovery rate ± standard deviation of 2,6-TDA and 2,4-TDA were 99.5±6.1% and 102.6±5.1%, respectively. When 25 μg/l of individual TDA standard was added to one of the control urine samples, the coefficient of variations of 2,6-TDA and 2,4-TDA determination were 5.7% and 6.3% (n=10), respectively.

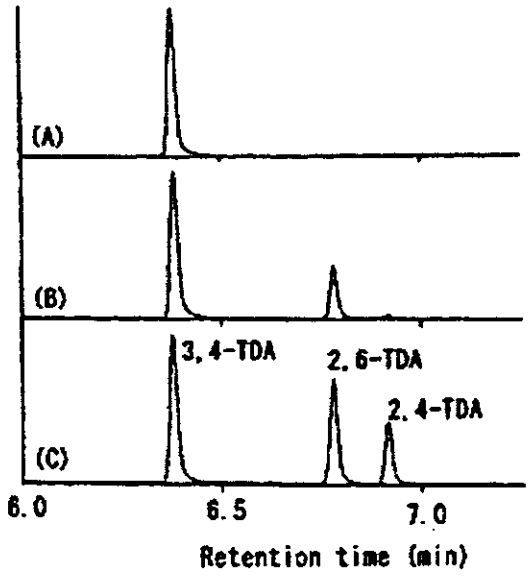

Fig. 1 Chromatographic separations of TDA isomers. **a** Control subject, **b** TDI worker, **c** standard TDIs. Concentrations of 2,6-TDA and 2,4-TDA in the worker were 29.1 μg/l and 3.2 μg/l, respectively, and those of standard TDAs were equivalent to 50 μg/l

Table 1 indicates correlation equations among TDA values obtained by various hydrolysis conditions (urine dilution and hydrolysis time). Some of the correlations are shown in Fig. 2. When 11-fold diluted urine was used, the 2, 6-TDA levels obtained by 1.5 h hydrolysis were approximately 46.1%, as compared to the correlation slope of those by 16 h hydrolysis (Fig. 2a). This indicates that prolonged hydrolysis increases the yield of hydrolysis product 2,6-TDA. However, shortening the hydrolysis time is favorable for the time-consuming procedure of TDA analysis, although the values are nearly or less than half the complete hydrolysis (16 h). 2,6-TDA levels obtained in 11-fold diluted urine were slightly higher than those in 1.1-fold diluted urine when the hydrolysis time was 1.5 h (Fig 2b). This finding indicates that urine dilution is slightly effective for the hydrolysis of TDA-forming compound. Similar results were obtained for 2, 4-TDA, although the levels were as low as one-tenth of 2,6-TDA (Table 1). Based on these results, hydrolysis time can be shortened to 1.5 h with 11-fold diluted urine.

Table 1 Correlation equation ($y=ax+b$) among TDA values (x and y) by various hydrolysis conditions [*A* 1.5 h hydrolysis (y) versus 16 h hydrolysis (x) with 11-fold diluted urine, *B* 11-fold diluted (y) versus 1.1-fold diluted (x) with 1.5 h hydrolysis urine, and *C* 1.5 h hydrolysis of 1.1-fold diluted urine (y) versus 16 h hydrolysis of 11-fold diluted urine (x)]. *a* slope, *b* intersects the *y*-axis, *r* correlation coefficient, x and y TDA levels (μg/l)

Isomer	Urine used	Hydrolysis	*a*	*b*	*r*
2,6-TDA	Post-shift urine (n=18)	A	0.477	–0.313	0.984
		B	1.120	0.389	0.973
		C	0.395	0.138	0.942
	Pre-shift urine (n=18)	A	0.424	0.071	0.929
		B	1.120	–0.232	0.985
		C	0.353	0.735	0.879
	Both urine (n=36)	A	0.461	–0.247	0.966
		B	1.130	0.025	0.977
		C	0.381	0.360	0.923
2,4-TDA	Post-shift urine (n=18)	A	0.396	–0.072	0.839
		B	1.320	0.201	0.902
		C	0.307	–0.225	0.952
	Pre-shift urine (n=18)	A	0.326	–0.227	0.642
		B	0.430	0.285	0.683
		C	0.511	–0.547	0.633
	Both urine (n=36)	A	0.378	–0.193	0.778
		B	0.695	0.322	0.713
		C	0.360	–0.260	0.723

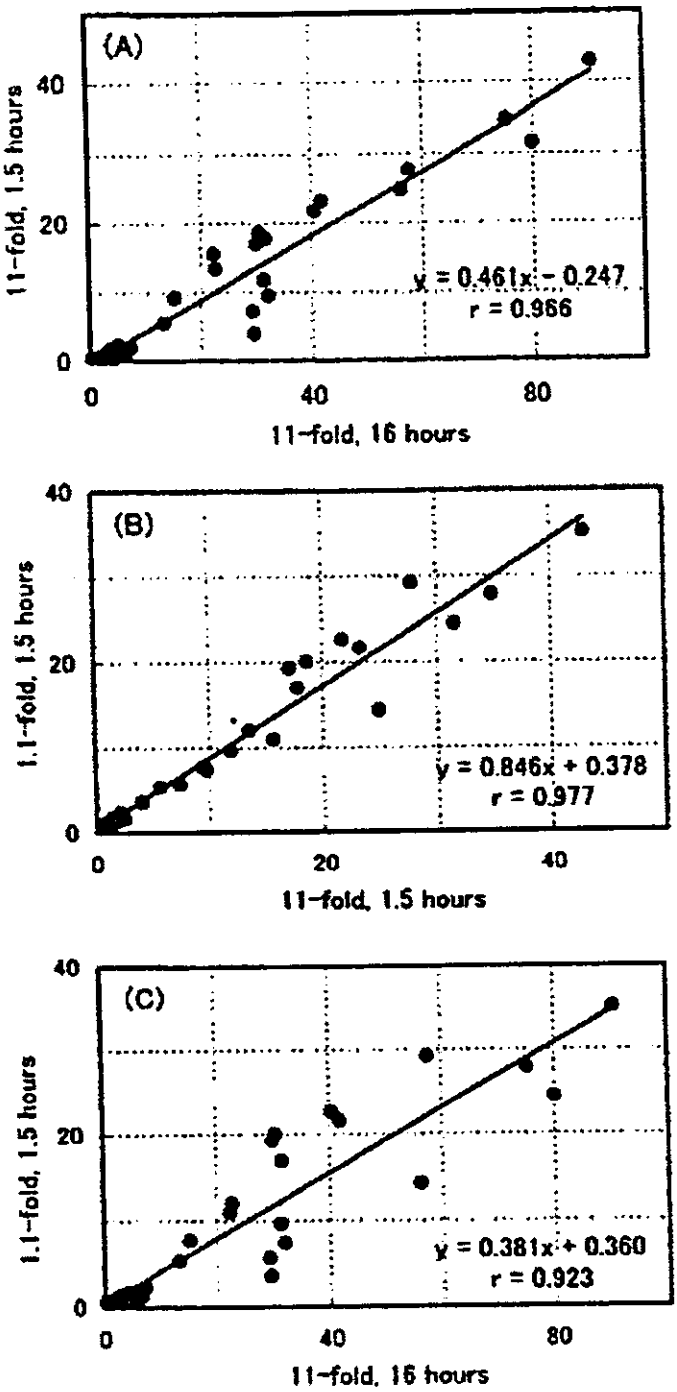

Fig. 2 Relationship between TDA values (x and y) by various hydrolysis conditions. Concentration of 2,6-TDA (μg/l) were compared among the hydrolysis condition of 11-fold or 1.1-fold diluted urine for 1.5 or 16 h. Both pre-shift and post-shift urine (n=36) was investigated together. **a** 1.5 h hydrolysis (y) versus 16 h hydrolysis (x) with 11-fold diluted urine. **b** 1.1-fold diluted (y) versus 11-fold diluted (x) with 1.5 h hydrolysis urine. **c** 1.5 h hydrolysis of 1.1-fold diluted urine (y) versus 16 h hydrolysis of 11-fold diluted urine (x)

When 11-fold diluted urine was hydrolyzed for 1.5 h, the TDA levels in post-shift urine were well correlated with personal exposure levels of TDI (Table 2). To investigate which is the better index for airborne TDI exposure, we investigated the relationships between airborne

TDI levels (x) and TDA levels in urine (y), both uncorrected and corrected concentrations. If the 2,6-TDI levels were corrected with creatinine or specific gravity (1.020), the correlation was improved, although the correlation in the 2,4-isomer was not improved because of low exposure levels. Creatinine correction showed the highest correlation coefficient (r=0.905) in the 2,6-isomer (Table 2 and Fig. 3b). TDAs in pre-shift urine were also correlated significantly with the personal exposure levels of TDIs, although the slope of the correlations in pre-shift urine was 60%–70% of that in the post-shift samples (Table 2). The correlation between 2,4-TDA and 2,6-TDA levels was significant (Fig. 4), although 2,4-TDA levels were less than one-tenth of 2,6-TDA levels. Thus, the levels of the 2,4-isomer were lower than those of the 2,6-isomer, not only in the urine but also in the work environment.

Table 2 Correlation equation ($y=ax+b$) between urinary TDA levels (y) and personal exposure levels of TDI (x). a slope, b intersects the y-axis, and r correlation coefficient. x personal exposure levels of TDI (ppb), y TDA levels in various correction of urine [*A* no correction (μg/l), *B* creatinine correction (μg/g creatinine), and *C* specific gravity (1.020) correction (μg/l)]

Parameter	Urine used	Correction	*a*	*b*	*r*
2,6-TDA vs 2,6-TDI	Post-shift urine (n=18)	A	10.160	–1.160	0.701
		B	6.600	–1.430	0.905
		C	8.130	–1.190	0.807
	Pre-shift urine (n=18)	A	6.090	–0.306	0.614
		B	4.930	–1.690	0.856
		C	5.920	–1.450	0.811
2,4-TDA vs 2,4-TDI	Post-shift urine (n=18)	A	8.330	0.231	0.835
		B	3.190	0.385	0.639
		C	5.340	0.334	0.782
	Pre-shift urine (n=18)	A	5.010	0.174	0.785
		B	2.900	0.124	0.847
		C	4.020	0.152	0.844

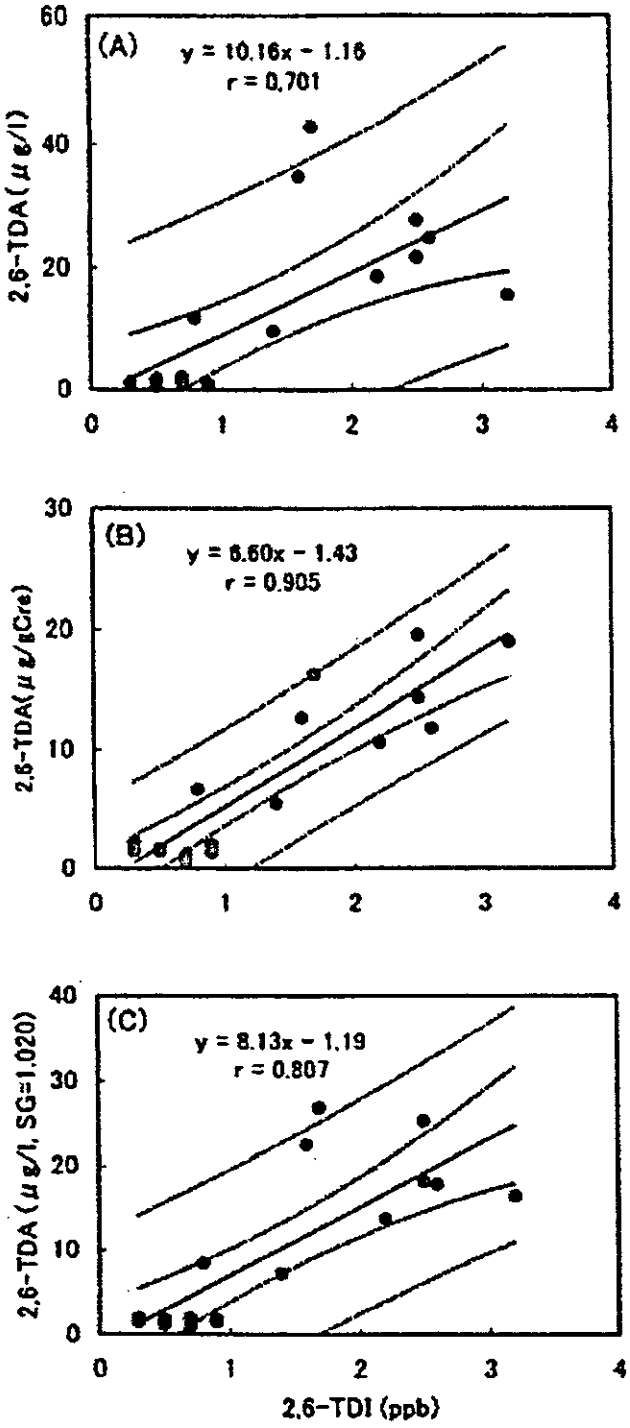

Fig. 3 Relationship between 2,6-TDA levels in post-shift urine (*y*) acquired by various corrections and personal exposure levels of 2,6-TDI (*x*) (*n*=18). **a** No correction (μ*l*), **b** creatinine correction (μ*l*/g creatinine), **c** Specific gravity (1.020) correction (μ*l*). *Solid lines* show regression lines. *Curves* (*dotted lines*) close to the regression line shows 95% confidence ranges of the regression line, and outermost curves (*dotted lines*) indicate 95% predictive intervals of individual values. *Cre* creatinine, *SG* specific gravity

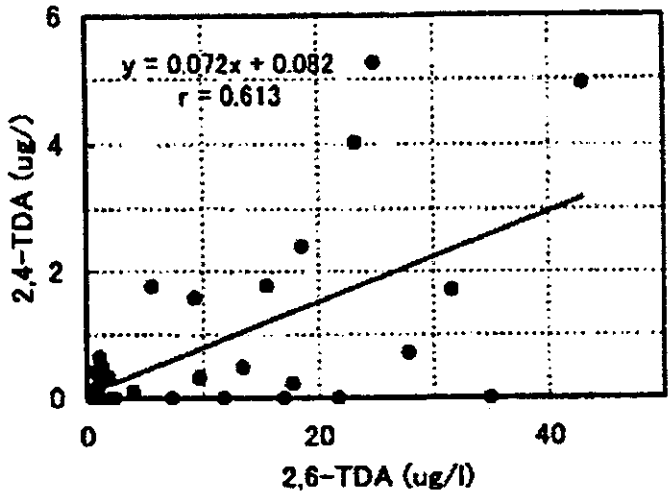

Fig. 4 Relationship between 2,4-TDA (*y*) and 2,6-TDA (*x*) in urine from workers. The data include both pre-shift and post-shift measurements (*n*=36)

Discussion

The most important improvement in the present method was the decreased volume of urine sample used (0.1 ml). More than 1 ml of urine (largest 3 ml) is used in the conventional methods (Brorson et al. *1991*; Lind et al. *1996*, *1997*; Maitre et al. *1993*; Persson et al. *1993*; Sandstrom et al. *1989*; Skarping et al. *1991*, *1994*). The decreased volume of urine used for the present analysis was attained by the use of DCM as the extraction solvent, which was highly effective for TDA extraction (Sakai et al. *2002*). Various conditions of hydrolysis, extraction and detection have been reported for the determination of TDA, so far (Table 3). Most analyses preferentially used toluene as the extraction solvent (Brorson et al. *1991*; Lind et al. *1996*, *1997*; Maitre et al. *1993*; Persson et al. *1993*; Sandstrom et al. *1989*; Skarping et al. *1991*, *1994*). However, the extraction efficiency of TDA with toluene was very much lower than that with DCM. We confirmed the high efficiency of extraction of TDA with DCM by a LC-MS method, which does not need the derivatization step (Sakai et al. *2002*). The extraction efficiencies of 2,6-TDA and 2,4-TDA by DCM were 76.2% and 74.6%, respectively. By contrast, those by toluene extraction were 27.1% and 26.9%, respectively (Sakai et al. *2002*).

Table 3 Comparison of various methods of TDA determination previously reported

Author	Volume of urine (ml)	Volume of acid (ml)	Acid	Dilution of urine (fold)	Final concentration of acid (mol/l)	Hydrolysis time (hours at 100°C)	Extraction solvent	D n
Sandstrom et al. (*1989*) and Brorson et	2	3	6 mol/l HCl	2.5	3.6	Overnight	Toluene	C

al. (*1991*)								
Maitre et al. (*1993*)	3	0.75	12 mol/l HCl	1.28	2.3	2	Toluene (twice)	C
Lind et al. (*1997*)	1	1.5	3 mol/l H_2SO_4	2.5	1.8	16	Toluene	C
Sakai et al. (*2002*)	1	0.1	18 mol/l H_2SO_4	1.1	1.8	1.5	Dichloromethane	L
Morita et al. (*2003*)	1	0.1	18 mol/l H_2SO_4	1.1	1.8	1.5	Diethyl ether	C
This issue	0.1	0.1	18 mol/l H_2SO_4	1.1	1.8	1.5/16	Dichloromethane	C

The conventional GC-MS methods required the use of deuterated isomers of TDA as internal standards (Brorson et al. *1991*; Lind et al. *1996*, *1997*; Maitre et al. *1993*; Persson et al. *1993*; Sandstrom et al. *1989*; Skarping et al. *1991*, *1994*). It is generally difficult to obtain deuterated internal standards, and it may not be possible to obtain the standard material in many laboratories, because they are very expensive. This may be one of the most important limitations of the application to biological monitoring. We have previously developed alternative substances as internal standards for TDA determination instead of deuterated ones (OSHA *1989*; Sakai et al. *2002*). In the present GC-MS method, we also eliminated the use of deuterated standards by substituting with 3,4-TDA as the internal standard.

We have previously demonstrated the effects of urine dilution and shortened hydrolysis time on the yield of hydrolysis. The data indicated that a long hydrolysis time and hydrolysis without urine dilution were not favorable for high yields of TDA (Sakai et al. *2002*). In many of the GC-MS methods, 2.5-fold diluted urine was hydrolyzed for quite long times (Table 3), such as 16 h or overnight (Brorson et al. *1991*; Lind et al. *1996*, *1997*; Maitre et al. *1993*; Persson et al. *1993*; Sandstrom et al. *1989*; Skarping et al. *1991*, *1994*). In the previous study (Sakai et al. *2002*). When we used 1.1-fold diluted urine, the yield of TDA hydrolysis reached the plateaux at approximately 4 h and decreased with prolonged hydrolysis time, especially at more than 10 h. Thus, the hydrolysis time should be less than 4 h if the 1.1-fold urine is used. When we used an 11-fold dilution of urine, 16-h dilution, showed the highest yield of TDA. In the present GC-MS method, we hydrolyzed 11-fold diluted urine for 1.5 h. The TDA levels after 1.5 h hydrolysis correlated well with those after complete hydrolysis (16 h), although the former levels were less than half of the latter. Both levels are comparable, from the correlation equation (Fig. 2a).

For practical purpose, it is difficult to hydrolyze all samples for 16 h because it is very time consuming. This might be one of the reasons why biological monitoring by TDA is not routinely used. TDA levels obtained by 1.5 h of hydrolysis can be converted by use of the correlation equation (Fig. 2a). When we need the exact TDA levels we can hydrolysis urine for 16 h. However, to monitor many workers, the TDA levels acquired by 1.5 h hydrolysis are sufficient to prevent TDI-induced respiratory disease.

Ours is the first report on the relationship between urinary TDA levels and personal exposure levels of TDI in the respective isomer in actual workers, although Maitre et al. (*1993*) presented a linear relationship between TDA and TDI levels in the form of combined isomers (r=0.910). They examined samples from only nine workers, and the range of total TDI (2,4-

TDA and 2,6-TDA) was 9.5–94 $\mu g/m^3$ (1.33–13.2 ppb). TDA levels ranged between 6.5 $\mu g/g$ creatinine (Cre) and 31.7 $\mu g/g$ Cre, and a TDI concentration of 38 $\mu g/m^3$ (5.34 ppb) corresponded to a TDA excretion of 18 $\mu g/g$ Cre. In the present study a 2,6-TDA level that corresponded to the 5.34 ppb of 2,6-TDI was calculated as 33.8 $\mu g/g$ Cre, if the 2,6-TDI level was extrapolated in the correlation equation in Fig. 3b. From this comparison, TDA levels in the present study were 1.9-times higher than those found by Maitre et al. (*1993*). The higher levels in the present study might be partly attributed to the fact that a more complete hydrolysis was archived by 11-fold dilution of urine and high concentration of acid (1.8 mol/l H_2SO_4 in the final solution) used for hydrolysis. Maitre et al. (*1993*) hydrolyzed 1.28-fold diluted urine with HCl (2.3 mol/l final concentration) for 2 h. The difference might be because there were fewer subjects (n=9) in their study (Maitre et al. *1993*). However, there should be further investigation at higher exposure levels than in our study and that of Maitre et al. (*1993*).

Brorson et al. (*1991*) carried out test-chamber exposure of TDI to two volunteers. From their data, the correlation equation can be calculated between TDI exposure levels (x ppb) and urinary TDA levels (y $\mu g/g$ Cre): y=8.29x–10.2 for the 2,6-isomer (n=6, r=0.942) and y=3.97x–1.33 (n=6, r=0.990) for the 2,4-isomer. These relationships between exposure and urinary metabolites are similar to our present data (Table 2): y=6.6x–1.43 (n=18, r=0.905) and y=3.19x+0.385 (n=18, r=0.639), respectively. In the correlation equation, the TDA levels at 5 ppb exposure of TDI in their study are near to those in our study, even though the exposure time in their study was only 4 h (half of that in our study). It might be because of the longer hydrolysis time (overnight) in their study (Brorson et al. *1991*) (see Table 3).

The threshold limit value [or occupational exposure limit OEL)] of TDI was set at 0.005 ppm for the prevention of respiratory disease such as asthma (ACGIH *2003*; JSOH *1992*). If we can determine the TDA level that corresponds to the TLV level of TDI (biological threshold value or BEI), it would be possible to prevent TDI-induced asthma by simply determining urinary TDA levels instead of determining airborne TDI, which require complex procedures. Furthermore, biological monitoring by TDA levels is useful for estimating the intake value of TDI by all pathways, including airways, skin, and digestive organs. In addition, we can prove the TDI exposure by determining TDA, even if the airborne TDI level had not been determined when the symptoms developed.

From Fig. 3a (uncorrected urine), it can be estimated that the TDI levels calculated from TDA might have had wide range, especially over the TLV of 5 ppb. It is better to use creatinine-corrected urine for estimating TDI levels, from the view of confidence limit (Fig. 3b). From the correlation equation, the 2,6-TDA level (corrected with creatinine) that corresponded to the TLV (or OEL) level of TDI (5 ppb) was calculated to be 31.6 $\mu g/l$. The relationship between TDA levels and asthma is now under investigation by questionnaire, health examinations, IgE test, and determination of TDA and TDI.

In the present study, TDA levels, even in pre-shift urine, also correlated well with the personal exposure levels of TDI, although the slope of the correlation in pre-shift urine was 60%–70% of that in the post-shift samples (Table 2). The data might suggest that each worker was constantly exposed to TDI in every day work and that the excretion of TDA due to the exposure during the previous day's work continued in the pre-shift urine. Urinary excretion of TDA was lasting for more than 20 h after the beginning of 4-h exposure to 2,4-

TDI and 2,6-TDI (Brorson et al. *1991*). In a study by Tinnerberg et al. (*1997*) within a flexible-foam plant, considerable concentrations of TDA in hydrolyzed urine were reported, even on Monday mornings before the start of the shift, and TDA levels in pre-shift urine gradually increased during the continuing working days. This finding might indicate that the TDA-forming compound is accumulated in the body, maybe in the form of protein adducts such as albumin (Brorson et al. *1991*; Tinnerberg et al. *1997*). Some part of the TDA found in pre-shift urine might be due to those protein adducts. In the data from Tinnerberg et al. (*1997*), 2,6-TDA levels in pre-shift urine were nearly half the levels of 2,6-TDA in the previous post-shift urine. These data are also compatible with the present findings. Since the exposure conditions in the work rooms were constant and continued for consecutive work days, TDA levels in pre-shift urine are relatively high and correlated with the exposure levels of the day, which might be not so different from those of the preceding day. However, in the work place where exposure levels are not constant from day to day, the relationship between TDA in pre-shift and post-shift urine will not be observed.

In our study we found that the levels of 2,4-isomer were less than one-tenth of the 2,6-isomer in both air (personal exposure) and urine (Fig. 3), although the plant used an 80:20 mixture of 2,4-TDI and 2,6-TDI. This remarkable difference between materials used in the factory and both levels in environment and urine might be attributable to the grater reactivity of the 2,4-isomer in the polymerization reaction. The 2,4-isomer was predominant in the air at the start of the polymerization process, and 2,6-isomer was the major isomer at the end of the process (Rando et al. *1984*). In the flexible-foam plant using an 80:20 mixture of 2,4-TDI and 2,6-TDI, the 2,6-TDA levels were more than ten-times higher than the 2,4-TDA levels in the urine of a worker processing the product (polyurethane resin blocks), while both isomer levels were nearly same in the urine of workers close to the foaming nozzle or at the beginning of the curing tunnel (Tinnerberg et al. *1997*). In the factory examined here, intermittent polymerization was carried out at many point in the large work room, resulting in 2,6-isomer predominance in the work environment and urine.

In summary, we have developed a simple and sensitive GC-MS method for the determination of TDAs in urine and have applied it to workers exposed to TDI. The TDA levels in post-shift urine were well correlated with personal exposure levels of TDI. The correlation was improved by correction with creatinine or specific gravity in the 2,6-isomer, but not in the 2,4-isomer because of low exposure level. From the correlation equation, the 2,6-TDA level (corrected with creatinine) that corresponded to the TDI level of 5 ppb was calculated to be 31.6 μg/l. TDAs in pre-shift urine also correlated significantly with the personal exposure levels of TDIs. The correlation between 2,4-TDI and 2,6-TDA levels was significant, although the levels of the 2,4-isomer were less than one-tenth of the 2,6- isomers in both air (personal exposure) and urine.

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Microbial metabolite of dimethylarsinic acid is highly toxic and genotoxic

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Abstract

Dimethylarsinic acid [DMA, $(CH_3)_2AsO(OH)$] causes cancer in the urinary bladder of rats. However, its mechanism of cancer or the ultimate carcinogenic form is not yet known. Rats administered dimethylarsinic acid excrete three unknown arsenic compounds (termed M-1, M-2, and M-3) in urine or feces, and these compounds are presumed to be produced by intestinal bacteria. *Escherichia coli* A3-6 isolated from a rat yielded two unknown arsenic compounds (M-2 and M-3) from dimethylarsinic acid and M-1 from trimethylarsine oxide (TMAO) in the presence of cysteine (Cys). Contents of M-2 and M-3 varied with cysteine concentration. The cytotoxicity and genotoxicity of the bacteria-free solution of dimethylarsinic acid or trimethylarsine oxide metabolized by *E. coli* A3-6 were studied using V79 cells. Dimethylarsinic acid (1 mM) metabolized by *E. coli* A3-6 in the presence of cysteine (1 mM) was highly cytotoxic (50% survival reduction concentration; 2.1 μM As) in V79 cells, and the toxic substance appeared to be M-2. The metabolite solution (at 2.5–10 μM total As) induced c-mitosis and tetraploids, and caused mitotic arrest, since it increased mitotic cells at the cytotoxic dose. The metabolite solution also significantly increased sister chromatid exchange (SCE) and chromosomal aberrations, most of which were chromatid gaps and chromatid breaks. A3-6 converted 96.1% of trimethylarsine oxide to M-1 in the presence of cysteine. This metabolite solution did not exhibit cytotoxicity or genotoxicity. The reported M-2 concentration in urine of rats administered levels of DMA via drinking water known to cause bladder tumors was sufficient to exhibit cytotoxic and genotoxic effects in urinary bladder. Thus, we hypothesize that intestinal bacteria play an important role in carcinogenicity of dimethylarsinic acid.

Keywords: Dimethylarsinic acid; *Escherichia coli*; SCE; Chromosomal aberration; Tetraploid; Mitotic arrest; C-mitosis

Introduction

Dimethylarsinic acid [DMA, $(CH_3)_2AsO(OH)$] is a tumor promoter and a complete carcinogen in the urinary bladder in rats (Li et al., 1998; Wanibuchi et al., 1996; Wei et al., 1999, 2002; Yamamomto et al., 1995). However, its mechanisms for the induction of the bladder cancer are not yet known (Kitchin, 2001). When arsenate is introduced into the mammalian body, it is reduced to arsenite, and then is methylated to monomethylarsonic acid (MMA) and DMA (Thompson, 1993). DMA is the major metabolite of inorganic arsenic in humans (Buchet and Lauwerys, 1987; Vahter, 1999).

DMA is a mitotic poison, causing mitotic arrest, inducing tetraploids (Eguchi et al., 1997; Endo et al., 1992) and c-mitosis (Iwami et al., 1997), and inducing abnormal spindles in mitotic cells (Kawata et al., 2001). DMA also induces aneuploids in mouse bone marrow cells (Kashiwada et al., 1998). Our previous studies using V79 cells and HL-60 cells showed that cysteine (Cys) enhanced cytogenetic toxicity of DMA including mitotic arrest, tetraploid formation, and induction of chromosomal aberrations (Kitamura et al., 2002) and also enhanced cytotoxicity and induction of apoptosis (Zhou et al., 2003). We found dimethylarsinous acid (DMAIII), a reduced form of DMA, in the mixture of Cys and DMA, and concluded that the enhancement by Cys of DMA-induced cytogenetic toxicity was due to the formation of DMAIII. Methylated trivalent arsenicals such as DMAIII and monomethylarsonous acid (MMAIII), intermediates reduced in the metabolic processing of DMA and TMAO, are more cytotoxic than arsenite (Cohen et al., 2002; Petrick et al., 2000; Styblo et al., 2000, 2002). They are very

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potent in a DNA nicking assay and in a single cell gel assay (Mass et al., 2001).

Yoshida et al. (1998) detected two unidentified metabolites, M-1 and M-2, in urine of rats after long-term oral administration of DMA, MMA, arsenite, or trimethylarsine oxide (TMAO). The amounts of M-1 and M-2 eliminated were higher in DMA-exposed rats than in rats treated with the other arsenicals. Another unidentified metabolite, M-3, was detected only in feces. These unidentified arsenic compounds might be produced in the intestinal tract since the production of M-1 or M-2 was larger after oral administration of DMA than after intraperitoneal injection (Yoshida et al., 2001).

Escherichia coli A3-6 was isolated from cecum of a rat chronically exposed to oral DMA. *E. coli* A3-6 produced M-2 and M-3 from DMA and M-1 from TMAO in GUM medium. A3-6 required Cys to metabolize DMA or TMAO to the unidentified compounds in bouillon medium. The retention time of M-2 in urine of DMA-exposed rats was consistent with M-2 in the sample obtained from the culture mixtures of DMA, Cys, and *E. coli* A3-6 on both cation- and anion-exchange LC-ICP-MS (liquid chromatography with inductively coupled plasma mass spectrometry) chromatograms. These results indicated that urinary M-2 in the rat was the same compound as M-2 produced by *E. coli* A3-6 (Kuroda et al., 2001; Yoshida et al., 2001). The chemical properties of M-2 were studied by LC-ICP-MS and LC/MS analyses. The molecular weight of M-2 was 154 Da and it was a sulfur-containing compound (Yoshida et al., 2003). The molecular weights of M-1 and M-3 were 152 and 170 Da, respectively. The molar ratios of As to S in M-1 and M-3 were confirmed to be 1:1 and 1:2, respectively (unpublished data).

Wanibuchi et al. (1998) suggested that the unidentified metabolite, M-2, might play an important role in the urinary bladder carcinogenesis induced by arsenic in rats since rats with urinary bladder cancer excrete a large amount of M-2 in urine (Li et al., 1998).

In the present study, the cytogenetic effects of DMA or TMAO metabolized by *E. coli* A3-6 were examined. We report that the metabolite M-2 is one of the most toxic arsenic compounds.

Materials and methods

Reagents. Sodium arsenite, sodium arsenate, MMA, DMA, and TMAO were obtained from Tri Chemical Lab. (Yamanashi, Japan). Iododimethylarsine for standard solution of DMAIII was obtained from Dr. W.R. Cullen (University of British Columbia, Vancouver, Canada). Iododimethylarsine in water is hydrolyzed to DMAIII (Mass et al., 2001). Bouillon medium was purchased from Nissui (Tokyo, Japan). Leibovitz-15 (L-15) medium was purchased from SIGMA-ALDRICH, Japan. Fetal bovine serum was obtained from ICN Biochemicals (Costa Mesa, CA, USA). Giemsa's solution was obtained from Merck, Darmstadt, Germany. Trypsin was purchased from Difco (Michigan, USA). Other chemicals (analytical grade) were obtained from Wako Pure Chemical Industry (Osaka, Japan).

Preparation of crude metabolized solution of arsenic compounds. *E. coli* A3-6 was precultured overnight in bouillon. The bacteria collected by centrifugation were suspended in 0.01 M phosphate-buffered saline (pH 7.4) of equal volume to the bouillon medium. To obtain metabolized solution of arsenic compounds, 1 mM of DMA or TMAO and various concentrations of Cys were added to the *E. coli* A3-6 suspension. After incubation at 37 °C for 1 or 6 h under aerobic condition, bacteria in the solutions were removed by centrifugation and filtration through an Ultrafree-MC (Millipore, MA, USA) with a cut-off value of 10 kDa, and the bacteria-free solutions were stored at −80 °C and used for experiments within 2 weeks. We confirmed that contents of arsenic compounds in the solutions were not changed in 1 month.

Cells and methods for cytogenetic study. V79 cells, which originated from Chinese hamster lung, were obtained from the Institute for Fermentation (Osaka, Japan). Cell number measured by hemocytometry after the cells were harvested. Vitality of cells was examined by staining with trypan blue.

To determine sister chromatid exchange (SCE), approximately 1×10^4/ml of V79 cells were plated in 35-mm-diameter Petri dishes with 5 ml L-15 medium and cultured for 24 h at 37 °C in a 5% CO_2 atmosphere. The medium was supplemented with 7% heat-inactivated fetal bovine serum and kanamycin sulfate (50 μg/ml). Various concentrations of metabolized solution of arsenic compounds and 1 μg/ml final concentration of 5-bromodeoxyuridine (BrdU) were added and the cells were cultured in the dark at 37 for 28 h in a 5% CO_2 atmosphere. Colcemid was not added except in the control experiments to avoid its mitotic blocking effect and to determine the net index of metabolized solution treatment. The cells were treated with a hypotonic solution of 0.075 M KCl and fixed with methanol–acetic acids (3:1). Metaphase figures were stained with 0.1 μg/ml Hoechst 33258, irradiated with a black lamp (15 W, 2 cm, 20 min) with SSC (0.3 M sodium chloride + 0.03 M citrate) and stained with 2% Giemsa's solution. The SCEs were counted in 50 metaphases.

For chromosome aberration experiments, the cells were exposed to metabolite solution without BrdU. The mitotic figures were stained with Giemsa's solution. When sister chromatids of a chromosome were separated from each other, mitotic figures were determined as c-mitosis. Two plates were used for a group in the experiments. Results are shown as averages of two plates. Data were statistically analyzed using Student's *t* test or chi-square test.

Liquid chromatography with inductively coupled plasma mass spectrometry. A Model HP4500 ICP-MS (Hewlett-Packard, DE, USA) was used for arsenic detection. The operating conditions for ICP-MS were established in accor-

dance with those reported by Inoue et al. (1994). A Model IC (Yokogawa Analytical Systems, Tokyo, Japan) was used for separating arsenic species. For separations of arsenic compounds, two separation modes, cation and anion exchange, were used. The cation mode experiment, using a Shodex RSpak NN-614 column (150 × 4.6 mm i.d.) packed with cation-exchange resin (Showadenko, Tokyo, Japan), was performed under the following conditions: mobile phase 5 mM HNO_3–6 mM NH_4NO_3, flow rate 0.8 ml/min, and injection volume 50 µl. The anion mode experiment, using an Excelpak ICS-A13 column (75 × 4.6 mm i.d.) packed with anion-exchange resin (Yokogawa Analytical Systems), was performed under the following conditions: mobile phase 3 mM NaH_2PO_4 at pH 6 with NaOH, flow rate 0.8 ml/min, and injection volume 50 µl. A guard column of the same packing type was used for analysis. An outlet from the separation column was directly connected to the nebulizer of the ICP-MS using an ethylenetetrafluoroethylene tube of 0.3 mm i.d.

Determination of arsenic metabolites by LC-ICP-MS. Stock standard solutions of sodium arsenite, sodium arsenate, MMA, DMA, and TMAO were prepared by dissolving each compound in pure water at a concentration of 100 mg As/l. Standard solution of DMAIII was prepared by dissolving iododimethylarsine in pure water and stirring for 30 min under a nitrogen atmosphere just before use. The final diluted aqueous standard mixtures were prepared from each stock standard just before use. The samples were thawed and diluted 50-fold with distilled water just before measurement by liquid chromatography with inductively coupled plasma mass spectrometry (LC-ICP-MS). To obtain precise measurements, 1 mg/l of germanium solution was used as the internal standard for ICPMS; the internal standard was added to the eluate from LC through a mixing joint before introduction to the ICP-MS. The ICP-MS detection mass was set to *m/z* 75 ($^{75}As^+$), *m/z* 72 ($^{72}Ge^+$), and *m/z* 77 ($^{40}Ar^{37}Cl$). The ion intensity at *m/z* 77 was of diagnostic value only in the examination for the possible occurrence of $^{40}Ar^{35}Cl^+$ interference at *m/z* 75. This method was linear in the range of 0.001–10 mg As /l, and the reproducibility (RSD) for 0.01 mg As/l standard arsenic compound was about 2%.

Results

Arsenic compounds in metabolized solution of DMA

The effect of Cys on the production of DMA metabolites was studied by LC-ICPMS. DMA (1 mM) was incubated with various concentrations of Cys (1–7 mM) in the presence of *E. coli* A3-6. DMA was not metabolized by the bacteria in the absence of Cys. After a 1-h incubation, The addition of Cys (3 mM) decreased DMA to 32% and increased M-2 to 37%. In the case of Cys concentrations more than 3 mM, M-2 production was decreased. A slight amount of DMAIII appeared at 1 mM Cys (1.2%) or 3 mM Cys (6.9%). Cys (1–5 mM) increased M-3 in a concentration-dependent fashion. An unknown peak (30%) appeared with 7 mM Cys (Fig. 1A). After 6-h incubation, Cys (7 mM) decreased DMA to 0% and increased M-3 to 99.5%. M-2 concentration was highest (37%) at 1 mM Cys. DMAIII and the unknown peak disappeared (Fig. 1B). The cytotoxic and genotoxic effects of the DMA metabolites produced by 6 h of incubation were then studied using V79 cells.

Cytotoxicity of metabolized solution of DMA

V79 cells were exposed for 28 h to DMA metabolites produced after a 6-h incubation with various concentration

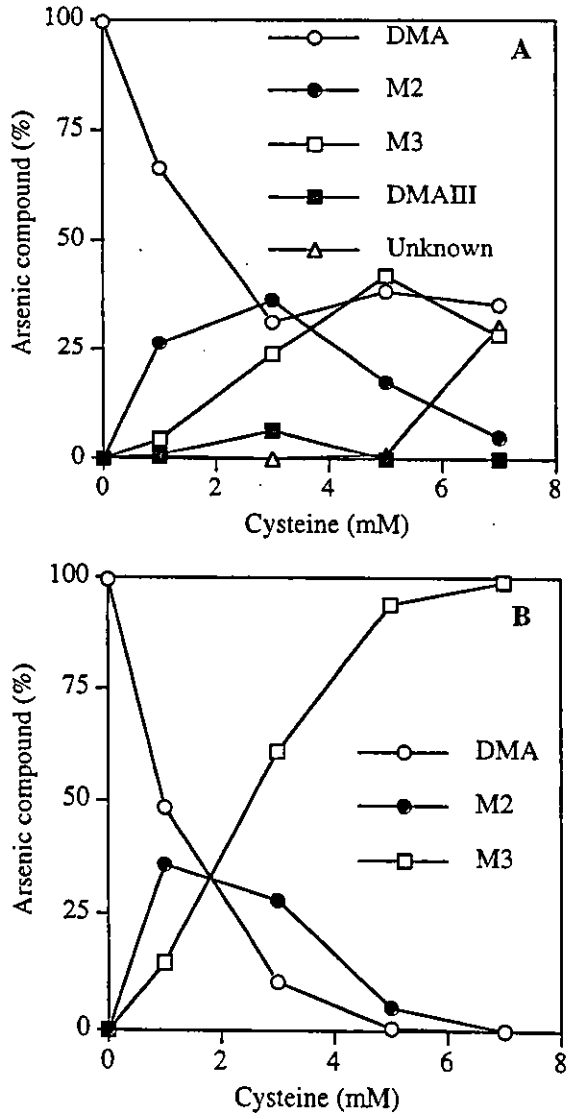

Fig. 1. Arsenic compounds in metabolized solution of DMA by *E. coli* A3-6 with various concentration of Cys. DMA 1mM and *E. coli* A3-6 were incubated in phosphate buffer with various concentration of Cys at 37 °C. (A) incubated for 1 h; (B) incubated for 6 h.

of Cys and cell survival was determined. The metabolites produced from a solution of 1 mM DMA and 1 mM Cys exhibited the strongest cytotoxicity and the metabolite solution produced by mixing DMA1 mM, and Cys 0 or 7 mM did not exhibit any toxic effect when used at 50 μM total As (Fig. 2). Amount of metabolite solution for 50% reduction of survival (IC_{50}) was calculated from Fig. 2. The IC_{50} values were 2.08, 4.03, and 10.71 μM As for Cys 1, 3, and 5 mM, respectively.

Information on toxic substance in a mixture is usually obtained by correlation between content of each substance and toxicity of the mixture. IC_{50} of the solutions was linearly correlated with content of M-2 (r = 0.999, P = 0.025, Fig. 3) and the contents of DMA or M-3 did not exhibit any correlation with cytotoxicity of the microbially metabolized solution (DMA; r = 0.806, P = 0.403, M3; r = 0.918, P = 0.259). These results strongly suggest that the cytotoxic substance in the metabolized solution of DMA was M-2.

Effects of DMA metabolite solution on mitosis

Chromosomal preparations of the V79 cells exposed for 28 h to the DMA metabolite solution produced by a 6-h incubation in the presence of various concentration of Cys were studied under a microscope. All the DMA metabolite solutions produced with Cys increased the numbers of mitotic cells in a concentration-dependent fashion (Fig. 4). The metabolites resulting from the incubation of

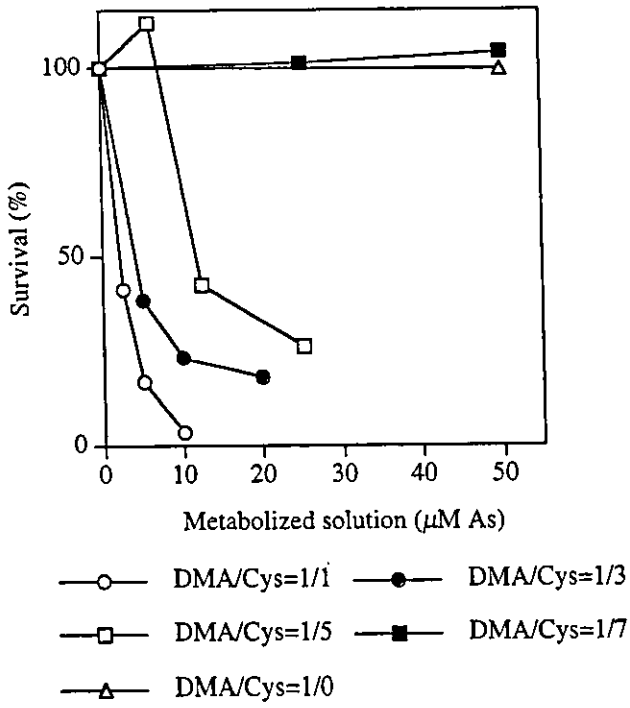

Fig. 2. Cytotoxicity of 6-h-incubated metabolized solution of DMA by *E. coli* A3-6 with various concentrations of Cys. V79 cells were treated with the solutions for 28 h. Survival was the average of two plates.

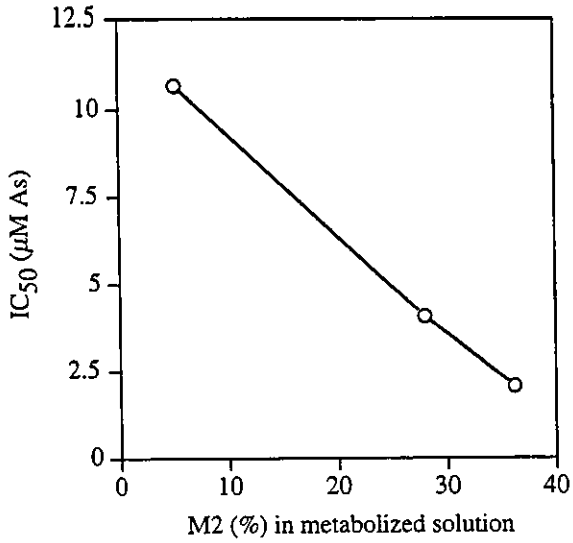

Fig. 3. Relationship between IC_{50} and M-2 content of 6-h-incubated metabolized solution of DMA by *E. coli* A3-6 with various concentrations of Cys. As concentration of IC_{50} was calculated from Fig. 2.

DMA (1 mM) and Cys (1 mM) were the strongest in increasing mitotic cells and mitotic cells became fewer as Cys concentration was increased. The DMA metabolite solution at Cys (1 mM) increased the frequency of mitotic cell to 4.4% at 10 μM As, which was more than twice the control rate (2.0%). At this concentration, the survival

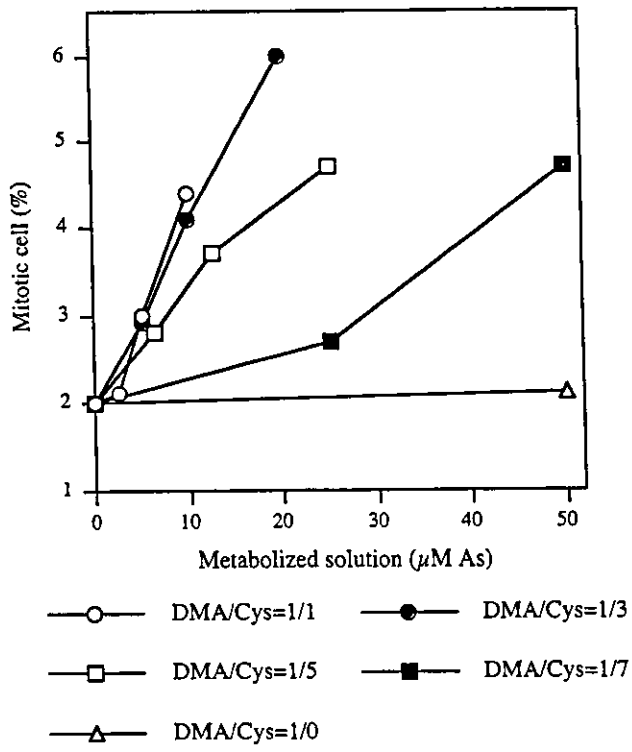

Fig. 4. Effects of 6-h-incubated metabolized solution of DMA by *E. coli* A3-6 with various concentrations of Cys on mitosis of V79 cells. V79 cells were treated with the solutions for 28 h. Frequency of mitotic cell was the average of two plates.

decreased to 4% of the control level. Mitotic figures were not observed at 20 μM As. These results suggested that the DMA metabolite solution caused mitotic arrest. The DMA metabolite solution produced without inclusion of Cys did not increased mitotic cells even at 50 μM As.

Amount of each DMA metabolite solution required for doubling the frequency of mitotic cells was calculated from Fig. 4. The values were 8.7, 9.6, 16.3, and 41.3 μM As for DMA metabolite solutions produced by incubation with 1, 3, 5, and 7 mM Cys, respectively. The value was exponentially correlated with M-2 content as shown in Fig. 5 (r = 0.999, P = 0.0008) and was not correlated with M3 or DMA (M3; r = 0.691, P = 0.309, DMA; r = 0.979, P = 0.128).

Induction of tetraploid by DMA metabolite solution

More than 90% of the mitotic figures in the control had 22 chromosomes and a mitotic figure of which chromosome number was 40–46 was determined as a tetraploid. The DMA metabolite solutions produced with Cys (1–5 mM) induced tetraploids in a concentration-dependent fashion (Fig. 6). The metabolites produced by incubation of DMA (1 mM) and Cys (1 mM) were the most active in this regard. Tetraploid in the control cells was 1% and in those of the cells exposed to the DMA metabolite solution produced with Cys (1 mM) at 2.5, 5, and 10 μM As were 10%, 14.9%, and 25.5%, respectively. The increases in tetraploids became weaker as the Cys concentration used to produce DMA metabolites was increased. The frequency of tetraploid cells appeared to decrease when the ratio of mitotic

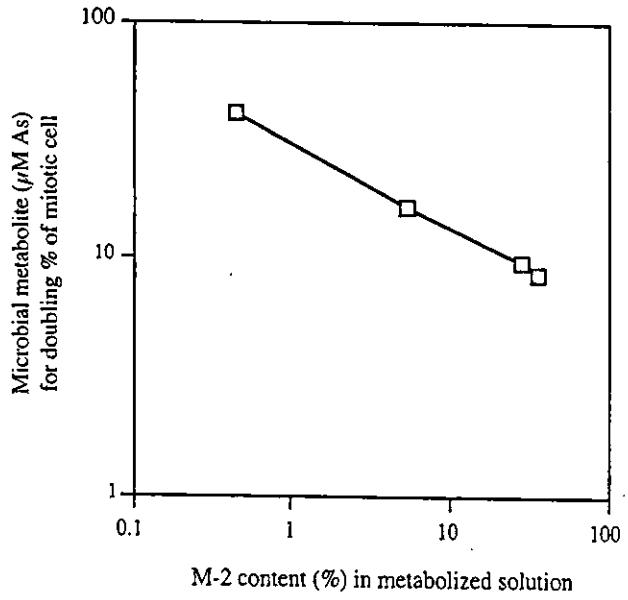

Fig. 5. Relationship between 6-h-incubated metabolized solution of DMA by *E. coli* A3-6 for doubling frequency of mitotic cell and M-2 content in metabolite solution. As concentration of metabolite was calculated from Fig. 4.

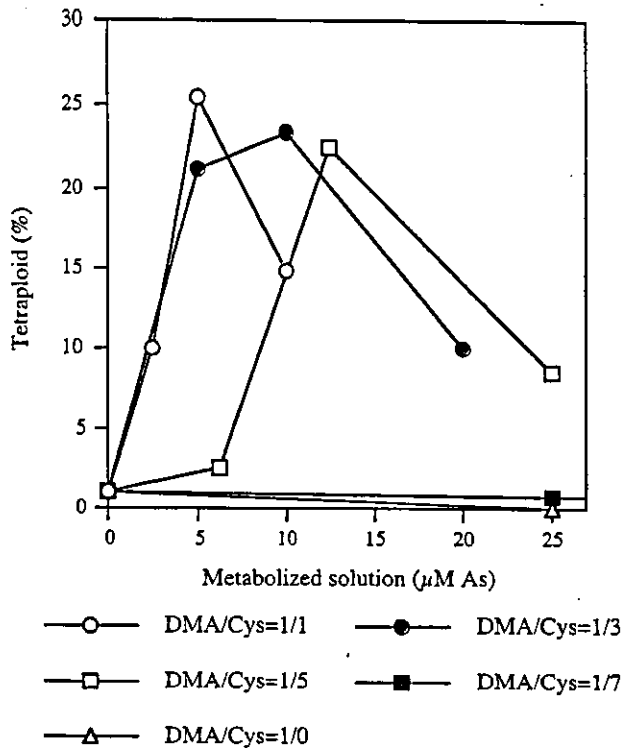

Fig. 6. Effects of 6-h-incubated metabolized solution of DMA by *E. coli* A3-6 with various concentrations of Cys on induction of tetraploid in V79 cells. V79 cells were treated with the metabolite for 28 h. Frequency of tetraploid was the average of two plates.

cells increased over 4%, twice that of control. The DMA metabolite solutions produced with 0 or 7 mM Cys did not induce tetraploidy at 25 or 50 μM As. These results suggested that M-2 in the DMA metabolite induced tetraploidy as well as mitotic arrest.

Induction of chromosomal aberration by DMA metabolite solution

Chromosomal aberrations were observed in the mitotic figures exposed to the microbial metabolite solution of DMA (1 mM) in the presence of Cys (1 mM). These results were summarized in Table 1. The rate of aberrant cells in the controls was 2%. The DMA metabolite solution at 5 μM As significantly increased the frequency of aberrant cells to 27%, 3-fold more than that induced by the DMA metabolite reduced by DMA alone at 100 μM As. Chromatid gaps were the most frequent aberration. Aberrant cells reached a rate 70% at 10 μM As of DMA metabolite. Chromatid breaks and chromatid gaps were the main types of aberrations. Mitomycin C, which was used for positive control, induced aberrant cells (34%) at 0.03 μM. The main types of aberrations here were gaps and chromatid exchanges.

c-Mitosis is considered an index of mitotic poisoning. More than 90% of the mitotic figures treated with the metabolized solution 5 and 10 μM As were c-mitosis. c-

Table 1
Chromosomal aberrations in V79 cells treated with metabolized solution of dimethylarsinic acid by *E. coli* A3-6 for 28 h

Chemical	c-mitosis (%)	Type of aberration					Total
		ctg	ctb	cte	csg	dic	
Control[a] 1/10 (v/v)	–[b]	1/1	0/0	0/0	1/1	0/0	2/2
Control[c] 100 μM As	71	7/8	2/3	0/0	0/0	0/0	9*/11*
Metabolized[d] solution 5 μM As	94	22/31	8/10	0/0	0/0	1/1	27**/42**
Metabolized[d] solution 10 μM As	97	54/149	54/113	1/1	0/0	0/0	70**/263**
MMC[e] 0.03 μM	–[b]	18/21	8/8	5/8	0/0	0/0	28**/34**

One hundred metaphases per group were observed. left/right, aberrant cell/ chromosomal aberration; ctg, chromatid gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break.
[a] Control was prepared with metabolized solution of A3-6 and Cys 1 mM and was added to V79 culture with 1/10 volume of culture medium.
[b] Treated with colcemid 0.1 μg/ml.
[c] Control was prepared with metabolized solution of A3-6 and DMA 1 mM.
[d] DMA 1mM and Cys 1mM were incubated with A3-6 for 6 h.
[e] Mitomycin C.
* $P < 0.05$, significantly higher than Control [1/10 (v/v)].
** $P < 0.001$, significantly higher than Control [1/10 (v/v)] and Control (100 μM As).

Mitosis was 7% in the control, whereas that of the metabolized solution without Cys was 71% at 100 μM As.

Induction of SCE by DMA metabolite solution

The DMA metabolite solution produced by incubation of DMA (1 mM) and Cys (1 mM) induced SCE in the range of 1 and 8 μM As (Table 2). It induced 0.60 ± 0.21 SCE per chromosome at 8 As μM in a dose-dependent manner. SCE at 16 μM As were not counted because the solution exhibited serious cytotoxic effects and most mitotic figures were once divided. Mitomycin C, which was used as a positive control, induced the same level of SCE (0.66 ± 0.20) at 0.003 μM.

Chromosomes per cell also increased in a concentration-dependent manner with the microbial DMA metabolite. Distribution of chromosome number in a mitotic figure of which sister chromatids were differentially stained is shown in Fig. 6. Mitotic figures in 94% of control had 22 chromosomes, and other mitotic figures (6%) were within one or two chromosomes of 22. A mitotic figure, which had 20–21 or 23–41 chromosomes, was determined as an aneuploid. Of the mitotic figures treated with the DMA metabolite solution (4 μM As), tetraploids were 6% and aneuploids were 8%, which were within one chromosome of 22. Tetraploids increased to 20% at DMA metabolite arsenic levels of 8 μM As and aneuploids increased to 12%. However, the increase of aneuploids by treatment of the microbial metabolite was not significant. These results suggest that the microbial metabolite of DMA specifically induced tetraploid (Fig. 7).

Table 2
SCE induction in V79 cells treated with metabolized solution of dimethylarsinic acid by *E. coli* A3-6 for 28 h

Metabolized solution (As μM)	SCE per cell	SCE per chromosome	Chromosome per cell
Control[a] 1/10 (v/v)	5.3 ± 2.4	0.24 ± 0.11	22.0 ± 0.4
Control[b] 10	5.3 ± 2.3	0.24 ± 0.10	22.1 ± 1.4
1	7.6 ± 3.3*	0.35 ± 0.15*	22.0 ± 0.3
2	9.2 ± 2.9*	0.42 ± 0.13*	22.1 ± 0.7
4	11.8 ± 4.9*	0.51 ± 0.19*	23.4 ± 5.3
8	16.2 ± 8.1*	0.60 ± 0.21*	26.8 ± 8.9*
MMC 0.03 μM	14.4 ± 4.4*	0.66 ± 0.20*	21.9 ± 0.3

SCE was counted in 50 metaphases of each groups. The metabolized solution was prepared after 6-h incubation of DMA 1 mM, Cys 1 mM, and *E. coli* A3-6. The metabolized solutions were prepared with DMA 1 mM, Cys 1 mM, and A3-6. MMC, mitomycin C.
[a] Control was prepared with metabolized solution of A3-6 and Cys 1 mM and was added to V79 culture with 1/10 volume of culture medium.
[b] Control was prepared with metabolized solution of A3-6 and DMA 1 mM.
* $P < 0.001$, significantly higher than control.

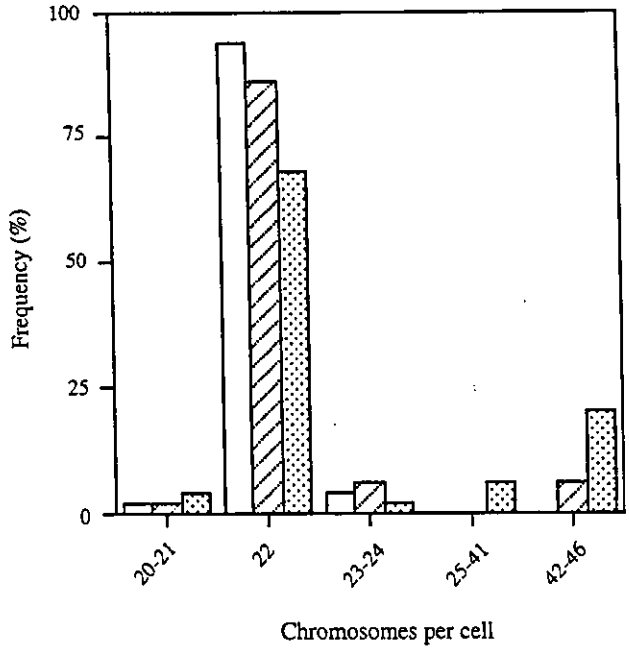

Fig. 7. Chromosome numbers in V79 cells exposed to 6-h-incubated metabolized solution of DMA 1 mM and Cys 1 mM by *E. coli* A3-6 for 28 h. Chromosomes in a mitotic figure, of which sister chromatids were differentially stained, were counted. White columns, control; striped columns, 4 μM As; dotted columns, 8 μM As of the solution.

Table 3
Toxicity of metabolized solution of V79 cells treated with metabolized solution of trimethylarsine oxide by *E. coli* A3-6 for 28 h

Metabolite (As mM)	M-1 (%)	Survival (%)	Mitotic cell (%)	Tetraploid (%)	SCE/cell	Aberrant cell (%)
Control[a] 1/10 (v/v)	0.0	100.0	2.9	1.5	4.8 ± 1.9	2
Control[b] 0.1	0.0	103.5	2.4	2.5	5.6 ± 2.0	1
0.1	96.8	102.9	3.5	2.0	5.5 ± 2.6	2

The metabolized solution of trimethylarsine oxide was prepared after 6-h incubation of TMAO 1 mM, Cys 1 mM, and *E. coli* A3-6.
[a] Control was prepared with metabolized solution of A3-6 and Cys 1 mM and was added to V79 culture with 1/10 volume of culture medium.
[b] Control was prepared after 6-h incubation of TMAO 1 mM and A3-6.

Effects of TMAO metabolite solution in V79 cells

E. coli A3-6 metabolized 96.1% of TMAO (1 mM) in the presence of Cys (1 mM) after incubation for 6 h, whereas the rest remained as unchanged TMAO. The TMAO metabolite solution did not exhibit cytotoxic or genotoxic effect in V79 cells at 0.1 mM As (Table 3).

Discussion

DMAIII found in metabolite solutions (Fig. 1A) might not be due to bacterial action because DMAIII is found in a mixture of DMA and Cys without bacteria (Zhou et al., 2003). The M-2 content in the 6-h-incubated metabolized solution was highest when DMA and Cys was equimolar. M-3 content increased with the concentration of Cys (Fig. 1). This result is reasonable because M-2 contains only one sulfur moiety (Yoshida et al., 2003) and M-3 contains two sulfur moiety (unpublished data). There are no reports of the direct participation of *E. coli* in the production of arsenothiol compounds. However, Tsao and Maki (1991) indicated that the reactive derivative, $(CH_3)_2AsSR$, was formed after the reduction of DMA by a thiol and the derivative bound to *Eco*RI methyl transferase by mercaptide exchange with a Cys residue located close to a tryptophan site.

The DMA metabolite solution produced with Cys was highly cytotoxic and M-2 was the probable main toxicant. M-2 content was linearly correlated with IC_{50} of the DMA metabolite solutions produced with various Cys concentrations (Fig. 3). However, IC_{50} of the microbial metabolite solution should be greater than 0 μM As, when M-2 content is extrapolated 100% and IC_{50} of DMA is about 1 mM in V79 cells (Kitamura et al., 2002), that is, M-2 content is 0%. Then a point (M-2 % = 0, IC_{50}= 1000 μM As) was added in Fig. 3. The relationship between M-2 content and IC_{50} appeared to be exponential as shown in the relationship between the value for doubling mitotic cell and M-2 content (Fig. 4).

It is important to compare our results with the microbial metabolites of DMA with those of trivalent arsenic because trivalent arsenic is more toxic than pentavalent. Regardless of the cell types, trivalent monomethyl arsenicals are more cytotoxic with IC_{50} values ranging from 0.4 to 5.5 μM. DMAIII derivatives are as cytotoxic as MMAIII species and more cytotoxic than arsenite in most cell types (Cohen et al., 2002; Petrick et al., 2000; Styblo et al., 2000, 2002). IC_{50} 5.4 μM of arsenite for V79 cells was estimated with the same method in the present paper (Eguchi et al., 1997).

Yamanaka et al. (1989) reported that volatile trivalent metabolites, dimethylarsine and trimethylarsine, were detected in the gas phase after DMA was added to cell suspensions of *E. coli* B in sealed tube. It is possible that the microflora present in the ceca are responsible for metabolism of arsenic compounds. The toxicity of complex of DMAIII with GSH (DMAIIIGS) was reported to be higher than that of DMA (Vega et al., 2001). Styblo et al. (1997) reported that complexes of MMAIII with GSH (MMAIII(GS)2) or with Cys (MMAIII(Cys)2) were the most potent inhibitors of yeast GSH reductase. These results strongly suggest that M-2 may be a trivalent sulfur-containing arsenic compound.

The DMA metabolite solution produced by incubation of DMA (1 mM) and Cys (1 mM) strongly induced c-mitosis, mitotic arrest, and tetraploid at μM As levels (Table 1, Figs. 4 and 5). This suggests that the microbial metabolite was a strong mitotic poison. DMA is also a mitotic poison but it is only active at millimolar concentration (Eguchi et al., 1997; Endo et al., 1992). DMA inhibits normal assembly of tubulin in vitro, induces multipolar spindles in mitotic cells (Kawata et al., 2001), and also inhibit tubulin's GTPase activity (Kawata et al., 2000). It is of interest whether the microbial metabolite of DMA exhibits the same activity as DMA.

Sodium arsenite induces SCE and chromosomal aberrations in different cell types. The aberrations found in many studies are chromatid gaps, fragmentations, endoreduplications, and chromosomal breaks (Gebel, 2001). However, tetraploidy induced by the DMA metabolite solution did not originate from endoreduplication. Sodium arsenite induces significant increases in chromosomal aberrations in human fibloblasts at 3.8 and 7.7 μM (Oya-Ohta et al., 1996). These results suggest that genotoxicity of the microbial metabolite with equimolar DMA and Cys was as strong as arsenite. We reported that M-2-induced cytotoxicity was decreased by the addition of SOD (Yoshida et al., 2003). This finding suggests that the production of reactive oxygen species may play an important role in cytotoxicity and the oxidative DNA damage by M-2.

Yoshida et al. (1998) reported that M-2 concentration in urine of rats administered DMA 100 mg/l As via drinking water for 7 months was 105 μM As. They suggested that M-2 was not produced through the metabolism of the rats because M-2 concentration was not affected by glutathione