Acta Biochimica Polonica

Vol. 50 No. 1/2003

291–296

www.actabp.pl

Communication

Detection of genotoxicity of atmospheric particles using a high-throughput microplate *umu*-test system★✪

Kunihiro Funasaka[1]✉, Masaaki Kitano[1], Akihiko Nakama[1], Taro Yoshikura[1] and Yoshimitsu Oda[2]

[1]*Osaka City Institute of Public Health and Environmental Sciences, Osaka, Japan,*
[2]*Osaka Prefectural Institute of Public Health, Osaka, Japan*

Received: 07 September, 2002; accepted: 31 January, 2003

Key words: *umu*-microplate test, S*almonella typhimurium* NM3009, S*almonella typhimurium* NM2009, atmospheric particles, genotoxicity, nitroarenes

A previously developed and highly sensitive *umu*-microplate test system based on the nitroreductase- and *O*-acetyltransferase-overproducing strain *Salmonella typhimurium* NM3009 and the *O*-acetyltransferase-overproducing strain *S. typhimurium* NM2009 was applied to the detection of genotoxic activity in atmospheric particles in urban areas using a relatively small sample load. The results showed that the test system was able to detect slight increases in induced genotoxicity in atmospheric particles and that genotoxicity was detected mainly in the fine fraction but also partially in the coarse fraction. The present sensitive microplate test system has potential for application to the screening of various other environmental samples.

Atmospheric suspended particles are one of the major air pollutants (Pope *et al.*, 1995; Reinchhardt, 1995). The particles originate from many processes such as diesel exhaust, industrial combustion and secondary conversion from gaseous pollutants (Friedlander, 1973). Especially, the urban particles contain thousands of chemicals including organic and inorganic components (Fraser *et al.*, 1998) and some of them produce mutagenic and car-

★Presented at the 32nd Annual Meeting of the European Environmental Mutagen Society, September 2002, Warsaw, Poland.

✪This research was supported by Grant-in-Aid for Young Scientists from the Japan Society for the Promotion of Science (KAKENHI: 13780441).

✉Corresponding author: Kunihiro Funasaka, Ph.D., Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennoji, Osaka 543-0026, Japan; tel.: (81 6) 6771 3282; fax: (81 6) 6772 0676; e-mail: Kunihiro.Funasaka@iphes.city.osaka.jp

Abbreviations: Me_2SO, dimetylsulfoxide; PTFE, polytetrafluoroethylene.

cinogenic effects (Goldsmith, 1980). It is, however, difficult to analyse most of the harmful chemicals in particulate samples because of the multiplicity of known and unknown chemicals present in the environmental complex mixture. Rapid biological screening methods are therefore useful for assessing human health risks.

Many researchers have used the Ames assay to examine the ability of histidine-requesting strains of *Salmonella typhimurium* to revert to prototrophic mutations, and the potent mutagenic activities of numerous chemicals have been reported (Ames *et al.*, 1975; Mortelmans & Zeiger, 2000). The Ames assay has also been used for the detection of mutagenicity in various environmental samples and novel field studies suggest that the kinds and the composition of carcinogenic substances present in environmental samples differ depending on the source of pollution (Daisey *et al.*, 1984; Matsumoto *et al.*, 1998). While the Ames assay is thus useful as one of the best short-term bioassay methods for detection of mutagenic activities in various known and unknown samples, it requires over 48 h and relatively large volumes of test samples.

In contrast, SOS/*umu*-test system has been developed for the rapid detection of DNA-damaging agents. In the system, the *umu* gene was cloned and a plasmid (pSK1002) carrying a *umuC′–lac′Z* fusion was constructed for the study of the regulation of *umu* gene expression (Shinagawa *et al.*, 1983). In this fusion gene, the *umu* operon is induced by DNA-damaging agents and regulated genetically by the *recA* and *lexA* genes. The induction of *umuC* gene expression could be monitored by measuring the cellular β-galactosidase activity produced by the fusion gene after DNA damage. Since the *umu* operon has a possibility to express mutagenesis more directly than other known SOS genes (Kato & Shinoura, 1977), the *umu* test system has been applied to rapid screening of environmental mutagens and carcinogens (Oda *et al.*, 1985).

Recently, a more sensitive SOS/*umu*-test system based on the nitroreductase- and *O*-acetyltransferase-overproducing strain *S. typhimurium* NM3009 and the *O*-acetyltransferase-overproducing strain NM2009 has been developed (Oda *et al.*, 1995). These strains have proved to be extremely sensitive towards the genotoxic activity of nitroarenes and aromatic amines, respectively (Yamazaki *et al.*, 1992; Oda *et al.*, 1993), because the bacterial strains deficient in nitroreductase or *O*-acetyltransferase are resistant to the mutagenic action of several nitroarene compounds (McCoy *et al.*, 1983). We have also developed a rapid and simple high-throughput *umu*-test system to be useful for the detection of nitroarenes and aromatic amines with quite small volume of chemicals (Oda *et al.*, 2001).

In the present report, we have attempted to apply the microplate SOS/*umu*-test system based on *S. typhimurium* NM3009 and NM2009 to the detection of nitroarenes and aromatic amines present in environmental atmospheric suspended particles in the urban Osaka (Japan). Our objectives were to apply the test system to actual particulate samples and to explore its applicability to other small-amount of environmental samples.

MATERIALS AND METHODS

In order to collect airborne particles, a low-volume Andersen cascade impactor (AN-200; Dylec Co., Tokyo) was operated continuously for approximately 10 days from Nov. 1 to 10 and from Nov. 10 to Nov. 20, 2000, at a site at *Hirano* in the eastern central area of Osaka, Japan. The sampler has the ability to collect separately on nine filter stages with aerodynamic diameters of more than 11 (stage 0), 7.0–11 (stage 1), 4.7–7.0 (stage 2), 3.3–4.7 (stage 3), 2.1–3.3 (stage 4),

1.1–2.1 (stage 5), 0.65–1.1 (stage 6), 0.43–0.65 (stage 7) and less than 0.43 mm (back-up stage). The filter (80 mm ϕ, QR100, silica fiber; Toyo Advantec Co., Japan) was preheated at 800°C for 2 h to avoid background contamination and kept in a room for over 48 h at 20°C and 50% relative humidity before use. At the end of each sampling, the filter of the sampler was removed, kept for over 48 h at 20°C and 50% relative humidity, weighed and then stored at −20°C before pretreatment. A quarter of each filter in two independent sampling days was cut into pieces and extracted with 100 ml of methylene chloride by ultra-sonication for 30 min at 0°C according to a previous method (Sweetman *et al.*, 1982; Ando *et al.*, 1991) with some modification. The extracted solution was filtered with anhydrous sodium sulfate using a cellulose filter (No. 5C; Toyo Advantec Co., Japan). The sample solution was then evaporated, dissolved in hexane, transferred to a handmade PTFE micro-concentration tube, concentrated almost to dryness, and thoroughly dissolved in 20 μl of Me_2SO in order to obtain a wide range dose-response curve. Aliquots of 4 μl of the sample solution were then carefully diluted with Me_2SO on a PTFE 96-well microplate. After incubation for 3 h in an incubation shaker (IS-963; Tomy, Japan) with 96 μl of exponential bacterial culture of *S. typhimurium* NM3009 without S9 mix, or with strain NM2009 plus S9 mix (4.8 : 1), the whole culture was transferred to a new polycarbonate microplate and bacterial growth was assesed as turbidity (A_{600}) for each well measured by microplate reader (Multiskan MS; Labsystems). Then, 10 μl of the culture was transferred to a new microplate and 90 μl of Z-buffer and 50 μl of 0.1% SDS solutions were added to the well, followed by 10 μl of chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, U.S.A.) solution (4 mg/ml in 0.1 M P-buffer) as a colorimetric reagent for β-galactosidase activity induced by *umu*C gene expression. The plate was then gently mixed and left to stand at 37°C for 15 min in the absence of S9 mix or for 5 min in the presence of S9 mix. After incubation, 100 μl of stopping solution (1 M Na_2CO_3) was added to each well and gently mixed and β-galactosidase activity (A_{570}) was measured. Relative β-galactosidase activity (units), which reflects relative *umuC* gene induction, was determined by A_{570}/A_{600}. The value of a blank filter was subtracted and averaged wide range dose-response curves were obtained for a couple of the independent sampling days. Finally, in order to evaluate the circumstances of the air involving particles, we calculated the average minimal reactive air volume and minimal particulate concentration defined as the dose showing the relative β-galactosidase activity double that induced by Me_2SO alone.

RESULTS AND DISCUSSION

Figure 1 shows average size distribution of particles collected in the sampling area from Nov. 1 to Nov. 10 and from Nov. 10 to 20, 2000. There seemed to be a temporal bimodal size distribution of particles in early wintertime in this urban area and the concentration of fine particles (less than 2 μm) was 1.6 times

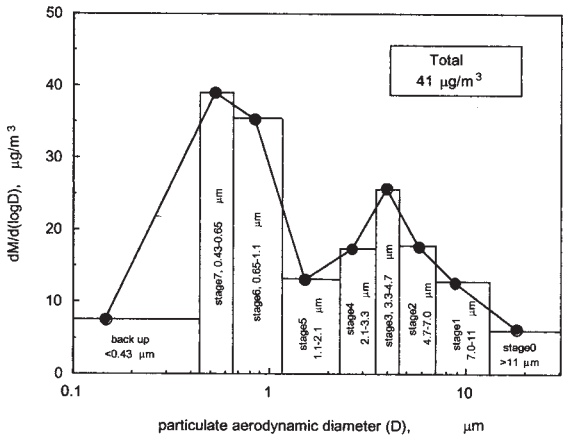

Figure 1. Average size distribution of atmospheric suspended particulate collected in early winter (from Nov. 1 to Nov. 10 and from Nov. 10 to Nov. 20, 2000) at *Hirano* in central (Osaka, Japan) using an Andersen cascade impactor.

higher than that of coarse particles (2 μm or more). Additional analytical results indicated that the fine particles included a large amount of carbonaceous components and water-soluble ionic species (not shown). We therefore speculate that the fine particles in the present sample are temporally affected by various direct anthropogenic activities including diesel exhaust (Ross *et al.*, 1987) and indirect secondary atmospheric conversion of gas to particles (Mizohata & Mamuro, 1980). The new microplate test system based on *S. typhimurium* NM3009 without S9 mix and NM2009 with S9 mix was applied to the samples fractionized by typical particle size.

Figure 2 shows relative β-galactosidase activity for particulate samples fractionized by particle size (indicated by stage number) as an average value within the two sampling periods, with plots drawn as a function of atmospheric air volume. The average value for relative β-galactosidase activity induced by Me_2SO alone was 0.61±0.17 (n=8). The fine particulate group (stages 5 to 7 and back-up filter) shows a markedly higher level of β-galactosidase activity than the coarse particulate group at the sampling air volumes of over 10 m^3. The results indicate strong SOS/*umu*-genotoxicity in fine particles and weak genotoxicity in coarse particles in the absence of metabolic activation. The urban atmospheric particulate thus possesses potent *umu*-genotoxicity present mainly in the fine fraction, where it originates from direct anthropogenic sources such as diesel exhaust particles, and partly in the coarse fraction, where it originates through indirect particle re-suspension (Nicholson, 1988).

The results for the same samples using *S. typhimurium* NM2009 with metabolic activation by S9 mix are shown in Fig. 3 in the same manner as in Fig. 2. The indirect SOS/*umu*-genotoxicity was not as pronounced as the direct genotoxicity shown in Fig. 2, but slight increases in β-galactosidase activity were again found in the fine and to a lesser extent in the coarse particle fractions. There seemed to be almost no SOS/*umu*-

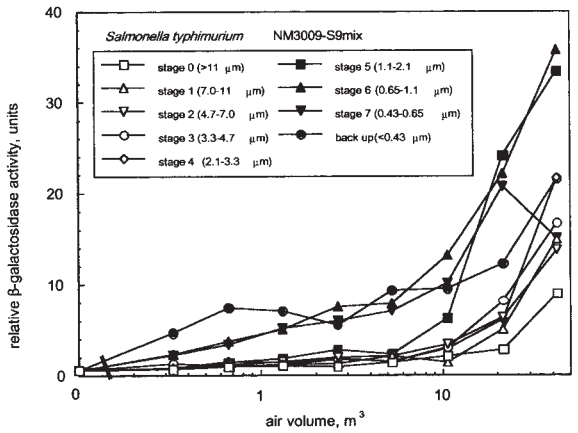

Figure 2. Relative β-galactosidase activity in size-fractionized particulate samples using high-throughput microplate *umu*-test with *S. typhimurium* NM3009 in the absence of S9 mix.

The value of a blank filter was subtracted and the plots represent the average of two independent sampling days as a function of air volume.

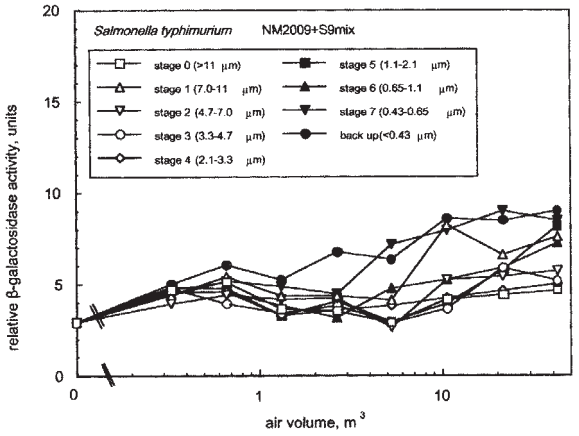

Figure 3. Relative β-galactosidase activity in size-fractionized particulate samples using high-throughput microplate *umu*-test with *S. typhimurium* NM2009 in the presence of S9 mix.

The value of a blank filter was subtracted and the plots represent the average of two independent sampling days as a function of air volume.

genotoxicity in the stage 0 (more than 11 μm), stage 2 (4.7–7.0 μm) and stage 4 (2.1–3.3 μm) fractions. The average relative β-galactosidase activity induced Me_2SO alone was 2.9 ± 1.3 (n = 8), 4.8 times higher than in direct SOS/*umu*-genotoxicity. We attributed this to the effect of the S9 mix itself in the assay with metabolic activation.

Minimal reactive air volume and minimal concentration, which represent atmospheric circumstances of the particles, are calculated and summarized in Table 1. The minimal concentrations of 1-nitropyrene and 2-aminoanthracene are also given as a positive standard. Minimal reactive air volume and minimal particulate concentration are low in the case of *S. typhimurium* NM3009 without S9 mix, with the lowest values commonly detected in the back-up filter fraction (< 0.43 μm). The results suggest that strong SOS/*umu* genotoxicity in the fine fraction depends on the presence of nitroarenes in the fine particles although detailed chemical analysis might be necessary. Minimal concentration in the fractionized particulate samples is higher than in the positive standard solutions both with and without metabolic activation. This may indicate that the suspended particles also contain other substances such as non-genotoxic chemicals originating in the earth's crust or elsewhere.

The present report shows that the new high-throughput microplate SOS/*umu*-test system with *S. typhimurium* NM3009 and NM2009 is able to rapidly detect slight increases in induced SOS/*umu* genotoxicity in atmospheric particles. Although it is necessary to investigate seasonal changes of particulate genotoxic capacity adding statistical analysis, the present test system is applicable to other environmental samples such as water, soil and mud with quite small sample amounts.

Table 1. Minimal reactive air volume and minimal particulate concentrations of the size fractionized particulate samples *umu*-microplate test system with *S. typhimurium* NM3009 without S9 mix and NM2009 with S9 mix.

The values represent the average of two independent sampling days. Minimal concentrations of 1-nitropyrene and 2-aminoanthracene are also shown in the bottom.

		S. typhimurium NM3009			*S. typhimurium* NM2009		
Sampling stage number	Size, μm	S9	minimal air volume, m^3	minimal conc., μg/ml	S9	minimal air volume, m^3	minimal conc., μg/ml
Stage 0	>11	–	4.5	0.49	+	none	none
Stage 1	7.0–11	–	2.2	0.22	+	6.6	0.66
Stage 2	4.7–7.0	–	0.95	0.12	+	none	none
Stage 3	3.3–4.7	–	0.25	0.040	+	17	2.7
Stage 4	2.1–3.3	–	0.45	0.061	+	none	none
Stage 5	1.1–2.1	–	0.55	0.081	+	21	3.1
Stage 6	0.65–1.1	–	0.15	0.048	+	20	6.5
Stage 7	0.43–0.65	–	0.15	0.042	+	3.8	1.1
back up	<0.43	–	0.05	0.028	+	0.55	0.15
1-nitropyrene		–		0.004			
2-aminoanthracene					+		0.009

none, not detected

REFERENCES

Ames BN, McCann J, Yamasaki E. (1975) *Mutat Res.*; **31**: 347–64.

Ando M, Tamura K, Katagiri K. (1991) *Int Arch Occup Environ Health.*; **63**: 297–301.

Daisey JM, Morandi M, Wolff GT, Lioy PJ. (1984) *Atmos Environ.*; **18**: 1411–9.

Fraser MP, Cass GR, Simoneit BRT. (1998) *Environ Sci Technol.*; **32**: 2051–60.

Friedlander SK. (1973) *Environ Sci Technol.*; **7**: 234–40.

Goldsmith JR. (1980) *J Envir Path Toxicol.*; **3**: 205–12.

Kato T, Shinoura Y. (1977) *Mol Gen Genet.*; **156**: 121–31

Matsumoto Y, Sakai S, Kato T, Nakajima T, Satoh H. (1998) *Environ Sci Technol.*; **32**: 2665–71.

Mizohata A, Mamuro T. (1980) *J Jap Soc Air Pollut.*; **15**: 225–33.

McCoy EC, Anders M, Rosenkranz HS. (1983) *Mutation Res.*; **121**: 17–23.

Mortelmans K, Zeiger E. (2000) *Mutat Res.*; **455**: 29–60.

Nicholson KW. (1988) *Atmos Environ.*; **22**: 2639–51.

Oda Y, Nakamura S, Oki I, Kato T, Shinagawa H. (1985) *Mutat Res.*; **147**: 219–29.

Oda Y, Yamazaki H, Watanabe M, Nohmi T, Shimada T. (1993) *Environ Mol Mutagen.*; **21**: 357–64.

Oda Y, Yamazaki H, Watanabe M, Nohmi T, Shimada T. (1995) *Mutat Res.*; **334**: 145–56.

Oda Y, Funasaka K, Kitano M, Nakama A, Yoshikura T. (2001) *Mutat Res.*; **483**: S183.

Pope CA, Thun MJ, Namboodiri MM, Dockery DW, Evans JS, Speizer FE, Heath CW. (1995) *Am J Respir Crit Care Med.*; **151**: 668–74.

Reinchhardt T. (1995) *Environ Sci Technol.*; **29**: 360A–4A.

Ross DS, Hum GP, Schmitt RJ. (1987) *Environ Sci Technol.*; **21**: 1130–1.

Shinagawa H, Kato T, Ise T, Makino K, Nakata A. (1983) *Gene.*; **23**: 167–74

Sweetman JA, Karasek FW, Schuetzle D. (1982) *J Chromatogr.*; **247**: 245.

Yamazaki H, Oda Y, Shimada T. (1992) *Mutat Res.*; **272**: 183–92.