Mutagenicity of Various Organic Fractions of Diesel Exhaust Particles

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Abstract

Treated with various organic fractions of Diesel Exhaust Particles (DEP), the Ames test with *Salmonella typhimurium* strains TA98 and TA100, and the mice micronucleus test were employed to study the mutagenic activity in the bacterial reverse mutation system, with and without a mammalian S₉ activation component, and the clastogenic activity in mice polychromatic erythrocyte (PCE) stem cells. Extracted ultrasonically with dichloromethane then using the acid and base separated reaction and column chromatography, DEP were divided into five organic fractions. They are the organic acid fraction (F1), the organic base fraction (F2), the aliphatic hydrocarbon fraction (F3), the aromatic hydrocarbon fraction (F4) and the polar fraction (F5). Results showed that an increase in the counted numbers of histidine revertants on the *Salmonella* TA100 and TA98 was observed with or without (S₉ mix), but these activities were more pronounced in the TA98 strains especially in the absence of the S₉ mix. These results suggest that the organic fractions of DEP contain mainly compounds with direct frame-shift mutagenicity. Positive results were also obtained from mice micronucleus assay. The frequency of mice bone marrow micronucleated polychromatic erythrocytes (PCE) was increased using this assay and it showed a definite dose-response relationship. The results suggest that various organic fractions could affect spindle fiber function or formation in mammalian cells. Compared with the results of different organic fraction, the effects of the F2, F4 and F5 were found to be stronger than those of other fractions. Based on the findings obtained in the Ames and micronucleus tests, DEPs have genotoxic effects in both of the test systems.

Key words: diesel exhaust particles, organic fraction, mutagenicity, Ames assay, micronucleus assay

Introduction

Diesel emission is one of the sources of air pollutants in environment⁽¹⁾. Diesel exhaust is a complex mixture of gases, vapors, and soot particles. The soot consists of respirable, chain aggregate, elemental carbon particles having a highly specific surface area to which a mixture of organic compounds is absorbed. Estimates indicate that as many as 18,000 different substances can be adsorbed onto diesel exhaust particles (DEP)⁽²⁾. The adsorbed material constitutes 15%-65% of the total particulate

mass and the organic fraction, which contains more than 450 compounds, including known mutagens and carcinogens such as polynuclear aromatic hydrocarbons (PHAs)⁽³⁾. DEP less than 5 μ m in diameter, if inhaled, can pass through the airways of the lung and be deposited in the bronchioalveolar region, which may be involved with the apparent damaging effects of DEP observed in experimental animals inhaling whole diesel exhaust. Those effects are possibly associated with the overloading of lung tissue which induces an inflammatory response, increased rates of cell proliferation, and wounding. In rodents these effects occur at high exposure levels after prolonged exposure (>12 months) to diesel exhaust particles. These processes are believed to play an important role in the carcinogenicity process^(4,5).

Several studies have suggested that the lung tumor response of rats to chronic DEP inhalation might be attributed to the initiating action of genotoxic metabolites of the particle-associated organic compounds. Hayakawa K, et al. demonstrated that 53% of the direct-acting mutagenic activity of DEP was attributed to Nitro-PAHs, and polycyclic aromatic hydrocarbons

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in fractions of DEP were considered as possible suppressors of Nitro-PAHs⁽⁶⁾. Other results obtained from assays in bacterial strains demonstrate that the mutagenicity of DEP is largely dependent on the aromatic content of the fuel, mutagenicity was greatly reduced when the aromatic content of the fuel was lowered by hydrogen treatment. No clear relationship was observed between the sulfur content of the fuel and suggested a low contribution of the high mutagenicity of DEP⁽⁷⁾. Micronucleus induction in V79 and CHO cells treated with diesel emission particles was studied, and the results indicate that most, if not all, genotoxic compounds associated with DEP can be expressed by indicating the relationship between the micronucleus rate and treated DEPs⁽⁸⁾.

In China more and more diesel engine vehicles are being used, with the emerging and rapid increase in the number of diesel engines, DEPs contribute significantly to air pollution in urban areas. The mutagenic activity of chemical substances was generally studied in a number of test systems using in vitro and in vivo techniques. The Ames test and mouse micronucleus assays are the most widely used screening systems in evaluating the mutagenicity of chemical substances in different test endpoints. To evaluate the mutagenicity of various organic fractions of DEP in China, with the support of the Chinese National Nature Science Foundation, the study was done to carry out the Ames test and in vivo mouse micronucleus assay to determine genotoxic effects induced by various organic fractions of DEP.

Materials and Methods

Sampling and Extraction: The DC-10A diesel selected was made in China and is more commonly used. The particulate phase was collected with a high volume air sampler (CYQ-60) with 1 m³/min of flow volume, with the diesel engine operating under idling conditions during most of sampling process, and was accelerated to the maximum speed once every 3 min. The particulate matter was adhered on glass filter when the diesel exhaust passed through fiber⁽⁷⁾. Sampling was done for 20 min, and the total DEP was determined by weighing the filter before and after sampling. The particulate phase was extracted ultrasonically using dichloromethane (CH₂Cl₂) as the solvent, and the extracting process was repeated for three times (15-min/per time) and the sediments were removed. Using the acid and base separated reaction, and column chromatography, the extractant was divided into five organic fractions, the organic acid fraction (F1), the organic base fraction (F2), the aliphatic hydrocarbon fraction (F3), the aromatic hydrocarbon fraction (F4) and the polar fraction (F5). Various fraction samples were then dissolved in a known volume of dimethylsulfoxide (DMSO) and kept at -20 °C until testing⁽⁹⁾.

Ames test: *Salmonella typhimurium* strains TA98 and TA100 were supplied by Dr. Ames' laboratory, University of California at Berkeley. Exogenous metabolic activation was done using liver postmitochondrial fractions (S₉) obtained from Aroclor 1254-induced Sprague-Dawley rats. Plate incorporation assays were carried out following the method of Maron and Ames⁽¹⁰⁾. All tests were made in triplicate plates in two independent experiments. Positive controls were included in all the experiments in order to confirm stain sensitivity and S₉ activity. Each sample was tested using three concentrations, and the test concentrations of the samples were expressed as original particle equivalent micrograms. S₉ was used as the metabolizing system in an

amount of 50 μl S₉ per plate. After 2nd of incubation at 37 °C, the colonies of histidine reversion on each plate were counted with an Artek model 880 automatic colony counter. Positive mutagenic response was assessed only when there was at least a doubling in the number of colonies as well as a dose-response effect.

Statistical analysis of the Ames test is suggested in the EPA GeneTox work group report for *Salmonella typhimurium* mutation assay⁽¹⁰⁾. The criteria used to determine a positive effect in strains TA98 and TA100 is as the following. If the solvent control value is within the normal range for the laboratory, a test article that produces a positive dose response over three concentrations, with the highest increase equal to twice the solvent control value, is considered mutagenic.

Mouse micronucleus assay: The assay was based on the procedures developed by Schmid et al⁽⁸⁾. Micronuclei are believed to be formed from chromosomes or chromosome fragments left behind during anaphase and scored during interphase because they persist. The mouse micronucleus assay was performed to investigate the possible chromosome damage induced by various organic fractions. In this experiment, male Swiss mice (provided by the laboratory animal center of Shanghai Medical University, 5 mice per treated group) weighing 18-20g were used. The various organic fractions were diluted with DMSO to prepare 3 concentrations containing 4, 12, 36 mg particles/ml, respectively. Each mouse was given a 0.1ml sample (ip). The mice were injected twice with an interval of 24h and sacrificed 6 h after the second injection. Cyclophosphamide (CP) at 80 mg/kg body weight was used as the positive control and the negative control group received the vehicle only. The bone marrow of the femur was smeared on a glass carrier. Slides were left to dry in air, fixed with absolute methanol, and stained with Giemsa and May-Grunwald stain solution⁽⁹⁾. The number of micronuclei in polychromatophilic erythrocytes (PCE) was counted.

The frequency of micronucleated cells in each treatment was based on 1000 cells calculated on each 5 mice per group. The results were expressed as the average number of micronucleated cells per 1000 cells with standard deviation, the total number of micronucleated cells in each treatment, and the relative micronucleus frequency of the treated group to the negative control.

Statistical analysis was based on a Poisson distribution of micronucleus rate using the method suggested by Amphlett and Deiw⁽¹¹⁾, the set of micronuclei frequencies among the controls is compared to the set for each treatment level. Increases above the negative control frequency that are significant at P<0.01 are considered indicative of an active agent.

Results

The composition results of organic fraction showed that the aliphatic hydrocarbon fraction (F3) and the polar fraction (F5)

Table 1 The composition of organic fraction of DEP

Fraction	Content (mg)	Composition (%)
F1	6.7	12.0
F2	9.0	16.0
F3	16.0	28.6
F4	9.1	16.3
F5	15.2	27.1
Total	52.0	100.0

Table 2 Histidine revertants/plate induced by different cocentrations of organic fractions of DEP in the presence and absence of *S*₉ mix (x ± SD)

Sample	Conc. (μg/plate)	Strains			
		TA100+ <i>S</i> ₉ mix	TA100- <i>S</i> ₉ mix	TA98+ <i>S</i> ₉ mix	TA98- <i>S</i> ₉ mix
DMSO	0.00	115.3 ± 3.1	112.7 ± 2.5	23.0 ± 1.0	26.7 ± 0.6
F1	250.00	115.5 ± 3.2	112.3 ± 2.1	26.3 ± 1.2	28.3 ± 1.2
	500.00	123.3 ± 2.1	124.3 ± 1.2	30.3 ± 0.5	32.0 ± 2.0
	1000.00	130.7 ± 2.3	131.7 ± 2.5	33.0 ± 1.5	35.0 ± 1.0
F2	250.00	118.3 ± 3.6	103.3 ± 2.6	21.3 ± 1.2	123.0 ± 5.2**
	500.00	123.5 ± 4.2	118.5 ± 1.2	25.6 ± 0.4	144.0 ± 4.5**
	1000.00	135.3 ± 5.1	130.3 ± 1.5	29.7 ± 1.5	172.7 ± 2.5**
F3	250.00	131.7 ± 5.6	116.3 ± 4.6	22.3 ± 1.2	97.0 ± 3.2**
	500.00	202.5 ± 4.2	131.0 ± 2.2	25.7 ± 2.5	125.5 ± 5.5**
	1000.00	226.3 ± 5.1	146.3 ± 4.1	34.0 ± 1.5	134.7 ± 8.5**
F4	250.00	112.7 ± 2.6	115.3 ± 2.6	36.3 ± 1.2	112.0 ± 4.2**
	500.00	115.5 ± 4.2	131.5 ± 3.2	38.0 ± 1.5	144.0 ± 6.5**
	1000.00	138.3 ± 2.5	138.3 ± 2.5	47.2 ± 2.5*	178.7 ± 2.5**
F5	250.00	117.3 ± 2.6	114.7 ± 2.1	32.3 ± 1.5	136.7 ± 6.2**
	500.00	128.5 ± 3.2	124.7 ± 2.5	35.3 ± 3.2	201.0 ± 6.5**
	1000.00	137.3 ± 2.1	152.3 ± 4.1	51.0 ± 2.0*	269.3 ± 7.5**
SA	1.00		1056.3 ± 34.5**		
2-AF	10.00	1102.0 ± 22.1**			1112.0 ± 20.1**
2,7-AF	100.00			980.0 ± 45.1**	

* ≥ 2-fold DMSO control, ** ≥ 3-fold DMSO control

X ± SD: Average number ± Standard Deviation

SA, sodium azide; 2-AF, 2-aminofluorene; 2,7-AF, 2, 7-aminofluorene

were greater than other fractions, 28.6% and 27.1% respectively, and the composition of the organic acid fraction (F1) was the least, only 12.0% (Table.1).

Ames test: In Table 2, in the experimental concentration ranges, there is a tendency towards increase in the colonies of histidine reversion on each plate, with the increase of treatment concentration. Samples showed mutagenic activities in both of the *Salmonella typhimurium* tester strains TA98 and TA100, but the activities were more pronounced in the TA98 strains than in the TA100 strains, especially in the “without mammalian activation” system (*S*₉ mix). The numbers of histidine revertants/plate induced by all organic fractions except F1 in the TA98 strains without *S*₉ mix, were obviously higher than those in the DMSO control (3-fold and more than the control). Comparing the histidine revertants/plate induced by various organic fraction of DEP, we found that the mutagenic activity (expressed by the average colonies of histidine revertant on each plate counted) of F2, F4 and F5 was higher than that of others.

Mice micronucleus assay: In Table 3, we found, with the increase of treatment concentration, the frequencies of micronuclei (the numbers of/1000 micronucleated PCE) formed in all 5 kinds of testers were significantly higher than of the DMSO control ($P < 0.05$ and $P < 0.01$) and were obviously dose dependent. But the micronucleus rates induced in all 5 kinds of samples differ. Comparing the micronucleus rates of different test groups, we found that F2, F4 and F5 can induce the higher micronucleus rates than the other groups. (Table 3)

Discussion

The Ames *Salmonella*/microsome plate assay was done to evaluate the test article for mutagenic activity in a bacterial reverse mutation system with or without a mammalian *S*₉ activation component^[2]. The results presented in this paper show that various organic fractions of DEP have mutagenic effects in the Ames test and in the mice micronucleus test in vivo. In the Ames test, the mutagenic activity was expressed with the counted

colonies of histidine revertant on each plate. The various organic fractions showed mutagenic activity in both of the *Salmonella typhimurium* tester strains TA98 and TA100, but there was obviously higher mutagenicity in the TA98 strain, and without the *S*₉ mix, it was greater than with the *S*₉ mix. These results suggest that various organic fractions of DEP mainly contain the compounds of direct-acting frame-shift mutagenicity. The data of the micronucleus test demonstrate that the incidence of micronucleus induced by various organic fractions of DEP increased significantly, which suggest that all various organic fractions can induce chromosome aberrations or spindle defects. Comparing the results of different organic fractions, we found that the organic base fraction (F2), the aromatic hydrocarbon fraction (F4) and the polar fraction (F5) have higher mutagenicity and induce higher rates of micronucleus than the

Table 3 Results of the mouse micronucleus assay

Sample	Dose (mg/mouse)	Number of PCEs scored	No.of(/1000) micronucleated PCE	Relative frequency
DMSO		5000	2.6 ± 0.82	1.00
F1	0.4	5000	3.4 ± 0.54*	1.31
	1.2	5000	6.2 ± 1.26**	2.39
	3.6	5000	10.2 ± 1.46**	3.92
F2	0.4	5000	5.4 ± 1.45**	2.08
	1.2	5000	8.5 ± 1.67**	3.27
	3.6	5000	15.2 ± 1.02**	5.85
F3	0.4	5000	4.1 ± 1.24*	1.58
	1.2	5000	7.6 ± 1.14**	2.92
	3.6	5000	12.6 ± 1.60**	4.85
F4	0.4	5000	6.5 ± 1.50**	2.50
	1.2	5000	9.5 ± 1.42**	3.65
	3.6	5000	17.2 ± 1.52**	6.62
F5	0.4	5000	5.6 ± 1.20**	2.15
	1.2	5000	9.1 ± 1.02**	3.50
	3.6	5000	16.5 ± 1.32**	6.35
CP	80mg/kg body weight	5000	36.5 ± 2.21**	14.04

* $P < 0.05$, vs DMSO control; ** $P < 0.01$ vs DMSO control

CP: Cyclophosphamide; PCE: Polychromatophilic erythrocytes

other fractions.

MN may arise either from structural chromosomal aberrations that are not incorporated into daughter nuclei at the time of cellular division or from segregation errors as a consequence of spindle defects. They represent an established parameter of genomic alteration¹³⁾. Micronucleus induction can be a very simple sensitive assay for the genotoxic effects to the DNA damaging agent⁹⁾.

The study was done by using two test systems which involved different test endpoints. The results from in vitro tests (Ames tests) were further supported by the in vivo test (MN assay) based on our study. These results suggest that DEP contains the agents which have mutagenic activity in the bacterial reverse mutation system and can affect spindle fiber function or formation. DEP may exert potential mutagenicity involving a tumor-related ability. In this study, we found there was no statistical significance in the F1 group in the Ames test, but there were significant statistical effects in the Micronucleus test. This may be because of a difference of the test endpoints,

which in the Micronucleus test there was sensitivity to the toxic effects of DEP, but not in Ames test. Considering this point, we concluded that when studying the biological effects of DEP, the different test endpoint experiments should be employed, and especially, when using the mammalian test system. The study also suggests that we should pay more attention to the organic fractions such as the organic base fraction, the aromatic hydrocarbon fraction and the polar fraction when studying the mutagenic effects of DEP, and also in researching the purification of DEP. Especially, it is reported that the amount of nitro-PHA in DEP is relatively higher, and accounts for a large portion of the mutagenicity of diesel exhaust particles¹⁴⁾. Most recently, attention has been paid to the nitroarenes, which are considered likely to contribute to much of the mutagenic potential of diesel exhaust. The study of Yoshiki et al has shown that nitro-PHA in DEP have direct frame-shift mutagenicity¹⁵⁾. At present when researching the DEP, we suggest that more attention should be paid to the aromatic hydrocarbon fraction, especially to the biologic effects of the nitro-PHA of DEP.

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