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In Vivo Induction of Sister Chromatid Exchanges in Mice by Nitrosated Coal Dust Extract

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The genotoxicity of coal dust extract nitrosated with sodium nitrite ($NaNO_2$) was investigated in mice with the *in vivo* sister chromatid exchange (SCE) assay system. The SCEs in bone marrow cells of mice were examined following single and double oral dosings of coal dust extract, $NaNO_2$, and nitrosated coal dust extract. Coal dust extract and $NaNO_2$ separately did not cause significant increases of SCEs either in single or in double dosings. Nitrosated coal dust extract in single doses did not increase SCEs but in two doses significant increases in SCEs were observed ($P < 0.02$). The mutagenicity of the same extracts was tested in *Salmonella typhimurium* with the Ames tester strain TA98. Coal dust extract was either non- or weakly mutagenic and $NaNO_2$ was nonmutagenic. The nitrosated coal dust extract caused pronounced increases in *his*$^+$ revertants both with and without rat liver S9 activation. These findings provide additional evidence that nitrosation of ingested coal dust which may occur in the stomach environment could be one of the factors leading to the higher incidence of gastric cancer in coal miners.

INTRODUCTION

Epidemiological studies have revealed that coal miners have higher gastric cancer mortality than nonminers (Stocks, 1962; Matolo *et al.*, 1972; Rockette, 1977). Recently, Ong *et al.* (1983) have proposed a hypothesis to explain the elevated incidence of gastric cancer among coal miners. According to this hypothesis coal dust is introduced into the stomach via lung clearance (Task Group Report 1966; Gross, 1971). Carcinogenic and/or mutagenic materials are formed in the acidic environment of the stomach either through intragastric nitrosation or other chemical interactions and are responsible for the increased incidence of gastric cancer in coal miners. To test this possibility, Whong *et al.* (1983) studied the mutagenic activity of coal dust extracts, with and without nitrosation, using Ames *Salmonella*/microsomal assay system (Ames *et al.*, 1975). They found that coal dust solvent extracts were non- or weakly mutagenic, whereas nitrosated coal dust extracts were highly mutagenic. Tucker *et al.* (1984) have reported induction of sister chromatid exchanges (SCEs) and chromosomal aberrations in human peripheral lymphocytes *in vitro* following nitrosated coal dust extract treatment. Increases in SCEs in Chinese hamster ovary cells and gene mutations in mouse lymphoma cells have also been found following exposure to nitrosated coal dust extract (Tucker *et al.*, 1984). However, nitrosated coal dust extract did not induce micronuclei formation *in vivo* in mice (Tucker *et al.*, 1984). Recent publications have called attention to the value of a short-term *in vivo* cytogenetic

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test, the SCE assay (Hollstein *et al.*, 1979; Kram *et al.*, 1979). The SCE analysis, with the exception of X rays and radiomimetic drugs, is more sensitive than conventional chromosomal aberration and micronucleus analyses in detecting genotoxic effect (Latt, 1974; Preston *et al.*, 1981). Because of the relative insensitivity of the chromosomal aberration test, subtle forms of genetic change, such as those detected by SCEs go unnoticed. The relative importance of the detection of such genetic changes has already been established (Carrano *et al.*, 1978; Krishna *et al.*, 1984a; Lockard *et al.*, 1981; Tezuka *et al.*, 1980). Using the SCE technique, studies with a wide variety of mutagens have shown a dose-related increase in the number of SCEs in different cells both *in vivo* and *in vitro* (Sandberg, 1982).

In this report the studies on genotoxic activity of coal dust extract, sodium nitrate ($NaNO_2$), and nitrosated coal dust extract have been extended to *in vivo* SCE induction in mice to generate additional information on the nitrosation of coal dust extract and its possible implication for gastric cancer in coal miners. The same extract was also tested for its genotoxicity in the Ames assay.

MATERIALS AND METHODS

Animals and chemicals. CD_1 male mice, 8–10 weeks old, weighing 25–30 g, purchased from Charles River Breeding Laboratories Inc. (Wilmington, Mass.) were used. A commercial diet (Purina Certified Laboratory Rodent Chow) and water were provided *ad libitum* throughout the period of animal holding and experimentation. Subbituminous coal from Sweet Water County, Wyoming was ground with a pestle and mortar and was used for extraction. $NaNO_2$ was purchased from Mallinckrodt, Paris, Kentucky. Cyclophosphamide (CPA), nicotinamide adenine dinucleotide phosphate, glucose 6-phosphate, 2,4,7-trinitro-9-fluorenone (TNF), and 2-aminoanthracene (2AA) were obtained from Sigma Chemical Company, St. Louis, Missouri.

Sample extraction and nitrosation. The extraction and nitrosation processes have been described by Whong *et al.* (1983). Basically, the coal dust (75 g) was extracted first by dichloromethane (DCM) and then by a 1:1 mixture of methanol and acetone (M + A). Each extract was concentrated to 0.5 ml, and then redissolved in 10 ml of dimethylsulfoxide (DMSO). Equal amounts of the DCM and M + A extracts were then mixed and an equal volume of $NaNO_2$ solution (15 mg/ml in DMSO) was added. The pH was then adjusted to 3.0 with 12 N HCl. The coal dust extract and the $NaNO_2$ solution alone served as controls and were also adjusted to pH ~3.0 under the same conditions. All the pH-adjusted solutions were incubated at 37°C for 3 hr with shaking (150 rpm) on a rotary shaker. Upon completion of incubation, the nitrosated and nonnitrosated coal dust extracts and $NaNO_2$ solutions were lyophilized and then redissolved in 20% DMSO and 80% corn oil. These were either used immediately or kept at −20°C until needed.

Sister chromatid exchange assay. Based on previous studies (Tucker *et al.*, 1984), five different concentrations of coal dust extract as well as nitrosated coal dust extract ranging from 5 to 80 g coal dust/kg were selected for toxicity studies. Also, four concentrations of $NaNO_2$, 9.75 to 70 mg/kg were tested for toxicities. Five animals were used for each concentration. The two highest doses of coal dust extract, 40 and 80 g/kg, caused 60 and 100% mortality, respectively. The same dosages of nitrosated coal dust extract caused 40 and 80% mortality. The

remaining three concentrations (5, 10, and 20 g/kg) did not show any toxicity. None of the $NaNO_2$ concentrations tested showed toxicity. Thus, concentrations of 10 and 20 g/kg for coal dust extract; 5, 10, and 20 g/kg for nitrosated coal dust extract and 35 and 70 mg/kg for $NaNO_2$ were chosen for SCE studies. For controls, only two doses of coal dust extract and $NaNO_2$ were tested as these were the two high doses used for the nitrosation process. The positive control chemical (CPA), the negative vehicle (corn oil with 20% DMSO), coal dust extract, $NaNO_2$, and nitrosated coal dust extract in different dosages were dosed through oral gavage to the experimental animals. In a second experiment, the compounds were dosed twice at an interval of 24 hr. Four mice per treatment per concentration were used for SCE analysis. The paraffin-coated bromodeoxyuridine (BrdU) tablets (50 mg, Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) were inserted under the skin on the flank 1 hr after the dosing of the test articles in 24-hr exposure and 1 h after the second dosing in 48-hr exposure (McFee *et al.*, 1983).

Animals were sacrificed through cervical dislocation after 3-hr colchicine (4 mg/kg) treatment. The bone marrow preparations for SCE analysis were made according to the established procedures (Latt *et al.*, 1981; Krishna *et al.*, 1985). Differential staining was achieved by a modified fluorescence plus the Giemsa method (Perry and Wolff, 1974; Goto *et al.*, 1978). To evaluate the cellular replication, the frequencies of the first, second, and third generation metaphases were determined in 50 consecutive metaphase cells from each animal. The replicative index (RI) was calculated using the formula

$$RI = \frac{1M_1 + 2M_2 + 3M_3}{100}$$

where M_1, M_2, and M_3 represent proportions of first, second, and third or subsequent division metaphases, respectively (Schneider and Lewis, 1981; Krishna *et al.*, 1985).

The results of SCE assays were analyzed for their significance using Student's *t* test over negative control values. For the cell cycle kinetics, the arc sin transformation was used and then a *t* test performed for each of the three divisions and RI.

Mutagenicity assay. Mutagenic activity was detected using the standard *Salmonella*/microsomal assay system (Ames *et al.*, 1975). Mutations were scored from histidine dependence to histidine independence. Determination of a positive mutagenic response was based on criteria recommended by Ames *et al.* (1975). The plate incorporation test with and without S9 activation in TA98 was conducted in the study. The liver homogenate of Aroclor-1254 (500 mg/kg body wt) pretreated male Wistar/Lewis rats was prepared according to Ames *et al.* (1975). For each treatment four plates per concentration were used.

RESULTS

SCEs in bone marrow cells following single administration of coal dust extract, $NaNO_2$, and nitrosated coal dust extract were slightly higher than controls (Table 1); these values however were not significantly different. Although there appears

to be a dose response, these data were not statistically significant. The data on two administrations of the same treatments are presented in Table 2. Coal dust extract and $NaNO_2$ induced slightly elevated SCEs over controls; however, the increase was not statistically significant. Nitrosated coal dust extract induced a slight but significant increase in SCEs over controls in a dose related manner ($P < 0.02$). The positive control compound, CPA, yielded large increases in SCE levels over all groups of animals.

In addition to assessing SCEs, the BrdU differential-staining technique was utilized to assess the effects of test compounds on cell replication. Cells which replicated once, twice, or three or more times in the presence of BrdU can be unequivocally identified. The data on the effects of different concentrations of coal dust extract, $NaNO_2$, and nitrosated coal dust extract with one and two administrations on cell replication *in vivo* are presented in Table 3. In general, the treatments did not induce cell cycle inhibition. The number of first, second, third, or subsequent division cells were in close approximation in all treatments and controls. On the average, the RI was approximately the same in all groups.

The extracts and the treatments used in the *in vivo* study were also tested for mutagenicity in *Salmonella typhimurium*, strain TA98 (Table 4). The coal dust extract was either non- or weakly mutagenic and $NaNO_2$ was nonmutagenic both with and without S9 activation. However, nitrosated coal dust extract produced relatively more *his*$^+$ revertants per plate, with a clear dose response both with and without S9 activation. In the highest concentration tested (90 mg coal dust + 0.35 mg $NaNO_2$) a toxic effect was evident in the absence of S9 activation.

DISCUSSION

The present study showed that nitrosated coal dust extract can induce slight but significant increases in SCEs in bone marrow cells of mice exposed twice

TABLE 1
SISTER CHROMATID EXCHANGES IN BONE MARROW CELLS OF MICE FOLLOWING *IN VIVO* EXPOSURE TO COAL DUST EXTRACT, SODIUM NITRITE, AND NITROSATED COAL DUST EXTRACT WITH A SINGLE DOSING

Treatment	Dose		SCEs/ chromosome	SCEs/cell ± SD[a]
	Coal dust (g/kg)	Sodium nitrite (mg/kg)		
Negative control[b]			0.056	2.23 ± 0.43
Positive control[c]			0.344	13.58 ± 1.02
Coal dust extract	10	0	0.075	2.95 ± 0.30
	20	0	0.079	3.13 ± 0.46
Sodium nitrite	0	35	0.073	2.91 ± 0.29
	0	70	0.090	3.58 ± 1.45
Nitrosated coal dust extract	5	17.5	0.077	3.03 ± 0.18
	10	35	0.082	3.22 ± 0.39
	20	70	0.101	3.99 ± 0.60

[a] Values represent variation among animals.
[b] Corn oil with 20% DMSO (approximately 0.30 ml per animal, depending on body weight).
[c] Cyclophosphamide, 10 mg/kg body weight.

TABLE 2
SISTER CHROMATID EXCHANGES IN BONE MARROW CELLS OF MICE FOLLOWING *IN VIVO* EXPOSURE TO COAL DUST EXTRACT, SODIUM NITRITE, AND NITROSATED COAL DUST EXTRACT WITH TWO DOSINGS

Treatment	Dose		SCEs/ chromosome	SCEs/cell ± SD[a]
	Coal dust (g/kg)	Sodium nitrite (mg/kg)		
Negative control[b]			0.088	3.50 ± 0.25
Positive control[c]			0.383	15.17 ± 0.93
Coal dust extract	10	0	0.098	3.88 ± 1.01
	20	0	0.097	3.85 ± 0.52
Sodium nitrite	0	35	0.105	4.15 ± 0.55
	0	70	0.108	4.30 ± 0.98
Nitrosated coal dust extract[d]	5	17.5	0.114	4.54 ± 1.37
	10	35	0.118	4.66 ± 1.03
	20	70	0.134	5.32 ± 1.33

[a] Values represent variation among animals.
[b] Corn oil with 20% DMSO (approximately 0.6 ml per animal (in total), depending on body weight).
[c] Cyclophosphamide, 10 mg/kg body weight.
[d] $P < 0.02$.

through oral gavage. A single dose treatment, however, did not induce a significant increase in the number of SCEs. It seems therefore that two dosings separated by a 24-hr interval allowed either sufficient time or concentration of mutagenic compounds to reach the bone marrow cells. Lack of response with nitrosated coal dust extract following one administration is in agreement with an earlier *in vivo* study using micronucleus assay (Tucker *et al.* 1984). A positive response in *in vivo* SCE induction following two administrations is comparable to *in vitro* studies in human lymphocytes (Tucker *et al.*, 1984). Even though Tucker *et al.* (1984) used two oral dosings of nitrosated coal dust extract, the results were negative in the micronucleus assay in mouse bone marrow cells. This may be due to different samples of nitrosated coal dust extract, different treatment conditions, or different concentrations of the sample. Also, the mechanism of formation of micronuclei is different from that of SCEs. The micronuclei formation in polychromatic erythrocytes involves either chromosomal breakage or failure of movement of a whole chromosome during division. However, SCE formation, even though the mechanism is not known, involves breakage, exchange, and repair of DNA. While agents which induce SCEs also commonly induce chromosomal damage (Latt, 1974; Perry and Evans, 1975), both qualitative and quantitative differences in the production of these alterations have been reported in a number of other cases (Gebhart, 1981). Nevertheless, the study reported here and that reported by Tucker *et al.* (1984) seem to indicate that the *in vivo* SCE assay is more sensitive than the micronucleus assay in detecting clastogenic activity of nitrosated coal dust extract in mice.

The data on cell kinetics following different exposures in a variety of conditions indicated nontoxicity of treatments. These results disagree with those of Tucker *et al.* (1984) who reported toxicity of coal dust extract in mice. This difference

TABLE 3
CELL CYCLE KINETICS IN MOUSE BONE MARROW CELLS FOLLOWING *IN VIVO* EXPOSURE TO COAL DUST EXTRACT, SODIUM NITRITE, AND NITROSATED COAL DUST EXTRACT WITH SINGLE AND DOUBLE DOSINGS[a]

Treatment	Dose		First div. cells[b] (%)		Second div. cells (%)		Third and subsequent div. cells (%)		Replicative index	
	Coal dust (g/kg)	Sodium nitrite (mg/kg)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Negative control[c]			9.0	12.5	91.0	84.5	0.0	3.0	1.91	1.90
Positive control[d]			6.0	6.5	94.0	93.5	0.0	0.0	1.94	1.93
Coal dust extract	10	0	6.0	8.0	88.5	89.5	5.5	2.5	1.99	1.94
	20	0	10.0	7.0	88.5	93.0	1.5	0.0	1.91	1.93
Sodium nitrite	0	35	12.0	11.0	88.0	89.0	0.0	0.0	1.88	1.89
	0	70	5.5	6.0	94.0	89.5	0.5	4.5	1.95	1.98
Nitrosated coal dust extract	5	17.5	8.0	11.0	90.5	83.0	1.5	6.0	1.93	2.00
	10	35	11.0	10.0	84.5	88.5	4.5	1.5	1.93	1.91
	20	70	9.5	9.0	90.5	85.5	0.0	5.5	1.90	1.96

Note. (1) = single dosing; (2) = two dosings.

[a] 50 consecutive metaphase cells were scored for the number of cell cycles traversed during the BrdU-implantation period in each animal (total of 200 metaphases per treatment).

[b] First-division cells represent cells that have undergone only one or no 'S' phase in the presence of BrdU.

[c] Corn oil with 20% DMSO.

[d] Cyclophosphamide, 10 mg/kg body weight.

can be attributed to the fact that these workers used only DMSO as the oral gavage vehicle which is known to be toxic. In our study the extract was first dissolved in a minimal amount of DMSO and then diluted appropriately with corn oil. Thus, it appears that the time, concentration, nitrosation of the chemical, and solvent vehicle are important factors in the *in vivo* genotoxicity of coal dust extract.

The results of the Ames *Salmonella*/microsomal assay confirm the findings of Whong *et al.* (1983) suggesting that the nitrosated coal dust extract used in *in vivo* study is indeed genetically active in inducing large numbers of *his*$^+$ revertants. It may be observed that the results in the bacterial assay may not be reflected in an *in vivo* situation where chemicals undergo deactivation.

In coal miners the exposure occurs over many years and the resulting cancers are gastric (Stocks, 1962; Matolo *et al.*, 1972; Rockette, 1977). Since the nitrosation reaction is hypothesized to occur in the stomach, this organ is the first to be exposed to the nitrosated coal dust chemicals (Ong *et al.*, 1983). The results reported here furnish additional evidence on the genotoxicity of nitrosated coal dust extract in an *in vivo* situation and its possible relation to gastric cancer in

TABLE 4
MUTAGENIC ACTIVITY OF COAL DUST EXTRACT, SODIUM NITRITE, AND NITROSATED COAL DUST EXTRACT IN *Salmonella typhimurium*, STRAIN TA98

Treatment	Dose per plate (mg)		Revertants per plate[a] ± SD	
	Coal dust	Sodium nitrite	−S9	+S9
Negative control[b]			29.0 ± 3.4	30.0 ± 1.6
Positive control[c]			470.7 ± 20.2	958.7 ± 24.6
Coal dust extract	11	0	27.7 ± 7.6	38.7 ± 3.0
	22	0	31.0 ± 5.3	39.0 ± 7.1
	45	0	42.0 ± 4.3	54.5 ± 4.8
	90	0	49.5 ± 6.6	61.0 ± 5.8
Sodium nitrite	0	0.04	30.5 ± 3.1	21.5 ± 2.6
	0	0.08	20.5 ± 1.7	27.0 ± 5.5
	0	0.17	29.0 ± 7.8	34.5 ± 4.2
	0	0.35	32.7 ± 7.7	32.7 ± 2.7
Nitrosated coal dust extract	11	0.04	693.2 ± 87.3	321.2 ± 5.1
	22	0.08	1120.0 ± 95.0	591.0 ± 22.1
	45	0.17	1517.5 ± 162.4	877.0 ± 37.3
	90	0.35	1023.7 ± 100.1	958.5 ± 50.0

[a] Results are mean values from four plates.
[b] 0.1 ml DMSO per plate.
[c] 2.5 μg 2AA per plate (with S9 activation) and 0.1 μg TNF per plate (without S9 activation).

coal miners. However, to properly evaluate its potential genetic and carcinogenic hazard to humans, chronic studies may have to be carried out. Also, genetic endpoints other than those studied may have to be included.

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