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Genotoxic Activity of Nitrosated Coal Dust Extract in Mammalian Systems

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The genotoxicity of coal dust extract nitrosated by $NaNO_2$ was investigated because of an elevated incidence of gastric cancer in coal miners. Human peripheral lymphocytes were used to determine the frequency of sister chromatic exchanges (SCE) and chromosome aberrations. Chinese Hamster Ovary (CHO) cells were also used to measure SCEs. The mouse lymphoma forward mutation assay comprised the final *in vitro* system, while the micronucleus test on mouse bone marrow cells was used as an *in vivo* assay. The SCE frequency in human lymphocytes increased from 9.2 per cell in untreated cultures to 31.6 per cell in cultures treated with 10.0 μl/ml of nitrosated coal dust extract (NCDE) ($P < .0001$). Chromosome aberrations were tested during two phases of the cell cycle; the results indicate that NCDE causes an increase in aberrations in each cell phase tested. The SCE frequency in the CHO system increased from 9.0 per cell in untreated cultures to 18.0 per cell in cultures treated with 3.3 μl NCDE per ml of medium ($P < .001$). In the mouse lymphoma system, the background mutation frequency was tripled at 2.0 μl NCDE per ml of medium. NCDE was not found to be active in the murine micronucleus assay. No consistent increase in genetic activity was observed with nonnitrosated coal dust extract alone or with $NaNO_2$ alone. Metabolic activation greatly reduced or eliminated genetic activity. These results indicate that nitrosated coal dust extract is genetically active in *in vitro* mammalian systems without metabolic activation. These findings suggest that the nitrosation of ingested coal dust may be responsible for the elevated incidence of gastric cancer in coal miners.

INTRODUCTION

Epidemiological studies both in this country and in the United Kingdom have revealed that coal miners have higher gastric cancer mortality than nonminers (Matolo *et al.*, 1972; Rockette, 1977; Stocks, 1962). Whong *et al.* (1983) postulated that coal dust breathed into the lungs is cleared by escalating, and via swallowing, is introduced into the stomach. Carcinogenic and/or mutagenic materials may then be formed in the acidic environment from coal dust and nitrite (an additive in processed meats). To investigate this possibility, Whong *et al.* (1983) have studied the mutagenic activity of coal dust solvent extracts, with and without nitrosation, from different types of coal by using the Ames *Salmonella*/microsome assay system (Ames *et al.*, 1975) and concluded that coal dust solvent extracts were either nonmutagenic or very weakly mutagenic with S9 activation. High

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mutagenic activities, however, were found when extracts of bituminous, subbituminous and lignite coal dusts were reacted with nitrite under acidic conditions. The mutagenic activity of nitrosated coal dust extract appeared to be independent of metabolic activation (Whong *et al.*, 1983).

Studies in our laboratory on the genetic toxicity of nitrosated and nonnitrosated coal dust solvent extracts were extended to several mammalian *in vitro* and *in vivo* assay systems. The genetic end points measured include gene mutations, micronuclei formation, sister chromatic exchanges and chromosome aberrations. In this paper we present the results of these studies.

MATERIALS AND METHODS

Test Compound

Subbituminous coal obtained from New Mexico was used in the study. The extraction and nitrosation processes have been described by Whong *et al.* (1983). Basically, the coal dust (50 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 sodium nitrite solution (30 mg/ml in DMSO) was added. The pH was then adjusted to 3.5 with 12N HCl. The coal dust solvent extract and the sodium nitrite solution alone were also adjusted to pH ~3.5, and served as controls in some of the experiments. All the pH-adjusted solutions were incubated at 37°C for 3 hr with shaking. The nitrosated and nonnitrosated coal dust solvent extracts were either used immediately or kept at −20°C until needed.

Assay Systems

Sister chromatid exchange assay with human peripheral lymphocytes. Heparinized human peripheral lymphocytes from two unrelated male donors (0.6 ml whole blood per 10 ml media) were cultured in the dark at 37°C in RPMI-1640 culture medium supplemented with 15% fetal bovine serum (FBS), L-glutamine (final concentration 2 mM), 1% penicillin–streptomycin (GIBCO), 0.1 ml phytohemagglutinin (PHA, GIBCO) and 25 μM bromodeoxyuridine (BrdU). The test compounds and positive and negative control substances were added at 24 hr, and the cultures incubated for an additional 48 hr. Mitomycin C (5 ng/ml) and DMSO served as the positive and negative controls, respectively. Colcemid (0.1 μg/ml) was added for the last 3 hr of incubation. The cells were swollen with 0.075 M KCl, washed three times in fresh fixative (methanol, acetic acid, 3:1), dropped onto slides, and air dried. Staining for detection of SCEs was achieved by a modified fluorescent plus Giemsa technique (Perry and Wolff, 1974; Goto *et al.*, 1978). Slides were stained for 15 min with Hoechst 33258 (5 μg/ml) in phosphate buffer (pH 6.8), exposed at a distance of 1 cm and 55–60°C to UV light at approximately 355 nm for 6–7 min, and then stained with 5% Giemsa for 10–15 min.

Chromosome aberrations with human peripheral lymphocytes. Human lymphocytes were cultured as described for SCEs. Genetic activity of nitrosated coal dust extract (NCDE) was tested during two different phases of the cell cycle, as

recommended by Preston *et al.* (1981). For G_0 testing, the test compound was added for 4 hr prior to PHA stimulation. The cells were then washed in Hanks' balanced salt solution, and cultured in fresh media with PHA for 52 hr. For S and G_2 testing, the test compound was added at 46 hr, and remained throughout the course of the 52-hr culture period. To ensure that only first division metaphases were scored, BrdU was included in the culture media. Staining of the slides was then achieved as described for SCEs.

Sister chromatid exchange assay with Chinese Hamster Ovary (CHO) cells. CHO cells were cultured at 37°C in McCoy's 5a medium supplemented with 10% FBS, L-glutamine, and antibiotics. The cells were seeded in 10 ml fresh medium at a concentration of 1×10^6 cells per 75 cm^2 approximately 24 hr prior to treatment. Cells were treated in the presence or absence of the S9 mixture. The S9 used in the experiments was prepared from the liver of Aroclor 1254 pretreated Fisher 344 male rats. To determine toxicity, cells were incubated for 24–26 hr after treatment, removed from the flasks with trypsin, and counted. The proportion of surviving cells was determined by trypan blue exclusion. The doses that were selected to score for SCEs included the dose that produces approximately a 50% reduction in cell survival, and/or a significant delay in cell cycle progression. To assay for SCEs in the nonactivation assay, cells were treated in growth medium. After 2 hr, 10 μM BrdU was added to the culture tubes and incubation continued in the dark for 26–32 hr. In the activation assay with S9, cells were treated in growth medium without FBS. After the 2-hr exposure period, the cells were washed twice with buffered saline containing 10% FBS. Fresh medium containing 10 μM BrdU was then added and incubation continued as in the nonactivation assay. Colcemid (0.1 μg/ml) was added for the last 2 hr of incubation and metaphase cells were collected by mitotic shake-off (Terasima and Tolmach, 1961). The cells were swollen and slides prepared and stained as described for human lymphocytes.

Micronucleus assay. Male Swiss–Webster mice of the CD-1 strain weighing 30–37 g were used. Each animal was given 0.15 ml/10 g body wt of the test compound by oral gavage as two equal administrations 24 hr apart. The test and control animals were treated in an identical manner. Cyclophosphamide was used as the positive control. The animals were starved overnight prior to treatment. Six hours after the second dose, the animals were sacrificed by cervical dislocation. Bone marrow cells in the femora were flushed with bovine serum into a 15-ml centrifuge tube and spun at 1000 rpm for 5 min. The supernatant was removed except for a few drops, and the pellet carefully mixed with the retained serum to yield a fine suspension. One small drop of the suspension was placed on each microscope slide, spread, and air dried. The slides were fixed for 5 min in absolute methanol, air dried, and then stained with Wright–Giemsa stain following the procedure of Schalm *et al.* (1975), but with the following modifications: the slides were stained for 3 min and the phosphate buffer (pH 6.4) was added for 20 min. After rinsing with distilled water and air drying, slides were placed in xylene for 5 min and mounted with cover glasses. The incidence of micronucleated cells per 1000 polychromatic erythrocytes was determined for each animal. The ratio of normochromatic to polychromatic erythrocytes was used as an indication of the toxicity of the compound upon the bone marrow cells.

Mouse lymphoma forward mutation assay: The Thymidine Kinase (TK) system. The procedure employed is based on that of Clive and Spector (1975). Briefly, cultures (TK +/− cells) exposed to the test chemical for 4 hr were washed and placed in growth medium for 2 or 3 days to allow recovery, growth and expression of the induced TK−/− phenotype. At the end of the expression period, 3×10^6 cells for each selected dose were seeded in soft agar plates with selection medium containing 50 μg BrdU/ml. Resistant (mutant) colonies were counted after 10 days of incubation. The number of viable cells was determined by plating a portion of the cell suspension in nonselective medium. The ratio of resistant colonies to the number of viable cells is the mutant frequency. The assay was run with and without S9 activation.

Statistical Analyses

The Student *t* test was used to compare the SCE frequency in test cultures with the negative controls. A statistical test is not generally employed for chromosome aberrations, although a doubling of the aberration frequency can be accepted as significant. The frequencies of micronuclei were subjected to the square root transformation as described by Snedecor and Cochran (1968). An analysis of variance was performed on the transformed data using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS). For the mouse lymphoma assay, mutant frequencies exceeding 150% of the concurrent background frequency were considered necessary to demonstrate mutagenesis.

RESULTS

In human lymphocytes, NCDE was found to be genetically active without metabolic activation. NCDE yielded similar and highly significant dose-responsive SCE rates in each of the two donors (Table 1). $NaNO_2$ alone was not genetically active, while nonnitrosated coal dust extract produced an elevated SCE frequency at the highest dose in one of the two subjects. Cell cycle delay was apparent. The negative control frequencies in this experiment, as well as those for the other experiments described here, are within the normal range for this laboratory.

NCDE also induced chromosome aberrations in human lymphocytes (Table 2). The G_0 phase appeared to be more sensitive to NCDE than the S and G_2 phases, although the difference may not be significant. Nonnitrosated coal dust extract, and $NaNO_2$ alone produced smaller increases. Cytotoxicity was apparent during G_0 testing at the highest concentration of NCDE, resulting in a reduced number of scoreable cells. Chromosome aberrations were also observed in CHO cells scored for SCEs, although specific testing of this cell line for aberrations was not conducted.

NCDE was genetically active in CHO cells without metabolic activation, but not in cells treated with S9 (Table 3). There was a highly significant dose-related increase in SCEs in cells exposed to NCDE at concentrations of 1 μl/ml and higher; cell cycle delay was also apparent.

The results of the micronucleus test are shown in Table 4. No significant differences in the number of micronucleated polychromatic erythrocytes were found

TABLE 1
SISTER CHROMATID EXCHANGES INDUCED BY NITROSATED COAL DUST EXTRACT IN HUMAN PERIPHERAL LYMPHOCYTES

Treatment	Donor	Number of SCEs[a]	SCEs per chromosome	SCEs/cell ± SE
DMSO (1%)	A	216	0.19	8.6 ± 0.7
	B	242	0.22	9.7 ± 0.8
Mitomycin C (5.0 ng/ml)	A	297	0.26	11.9 ± 0.7**
	B	375	0.33	15.0 ± 0.9**
NCDE				
0.33[b]	A	255	0.22	10.2 ± 0.7**
	B	267	0.24	10.7 ± 0.8**
1.00	A	333	0.30	13.3 ± 0.7**
	B	262	0.23	10.5 ± 1.0*
3.33	A	430	0.38	17.2 ± 1.0**
	B	416	0.37	16.6 ± 1.0**
10.00	A	777	0.69	31.1 ± 2.2**
	B	804	0.72	32.2 ± 2.1**
Coal dust extract				
3.33[b]	A	211	0.19	8.4 ± 0.7
	B	170	0.15	6.8 ± 0.6
10.00	A	301	0.27	12.0 ± 1.0**
	B	207	0.18	8.3 ± 0.8
$NaNO_2$ alone				
10.0[c]	A	262	0.23	10.5 ± 0.8**
	B	156	0.14	6.2 ± 0.7
30.0	A	194	0.17	7.8 ± 0.6
	B	182	0.16	7.3 ± 0.5

[a] Total for 25 cells.
[b] μl/ml.
[c] mg/ml. The concentrations of $NaNO_2$ are equal to those used in the two highest doses of NCDE.
* $P < 0.01$.
** $P < 0.001$.

in any of the treatment groups. However, nonnitrosated coal dust extract was found to be toxic at doses of 7 ml/kg body wt and higher, as nearly all the mice in these treatment groups died (data not shown). Mice treated with NCDE in an analogous manner survived, indicating that the nitrosation process converts cytotoxic (but not genotoxic) compounds to a less toxic form or forms.

The results from the mouse lymphoma forward mutation assay without metabolic activation are presented in Table 5. An elevated mutant frequency was consistently observed at 1 μl/ml, but not at the lower concentrations. Similar experiments conducted with metabolic activation also resulted in an elevated mutant frequency (data not shown).

DISCUSSION

The results of these studies indicate that nitrosated coal dust extract is genetically active *in vitro* without metabolic activation in mammalian assay systems. *In vitro* testing with activation was conducted using the mouse lymphoma forward

TABLE 2
CHROMOSOME ABERRATIONS INDUCED BY NITROSATED COAL DUST EXTRACTS IN HUMAN PERIPHERAL LYMPHOCYTES

Cell phase	Treatment	Number of cells scored	Type of aberration[a]								
			G	B	F	E	TD	R	D	O	Total
G_0	DMSO (1%)	100	1	2							3
	Mitomycin C (10.0 ng/ml)	100	10	4		2					16
	NCDE										
	0.33[b]	100	2	1					2		5
	1.00	100	8	3			3		1		15
	3.33	100	6	2	2		1	1		1	13
	10.00	75	6	4	8			1	3		22
	Coal dust extract										
	3.33[b]	100	2	1		1		4	1		9
	10.00	100		1							1
	$NaNO_2$										
	10.0[c]	100	3	2					2		7
	30.0	100	3		1	1		1			6
S, G_2	DMSO (1%)	100	5	3	1		1				10
	Mitomycin C (20.0 ng/ml)	100	21	3	2		1				27
	NCDE										
	0.33[b]	100	4	2	6			1	1	1	15
	1.00	100	7	2							9
	3.33	100	9	2		1	1				13
	10.00	100	11	3	1		2	1			18
	Coal dust extract										
	3.33[b]	100	10	3			1				14
	10.00	100	5	1			2				8
	$NaNO_2$										
	10.0[c]	100	3	2			1				6
	30.0	100	8	2			1				11

[a] Abbreviations used: G, gaps; B, breaks; F, fragments and acentric fragments; E, exchanges (translocations, triradials, quadriradials); TD, chromatid deletions; R, rings; D, dicentrics; O, other.
[b] μl/ml.
[c] mg/ml. The concentrations of $NaNO_2$ are equal to those used in the two highest doses of NCDE.

mutation assay, and SCEs in the CHO system. In the mouse lymphoma assay NCDE was less toxic, but was still mutagenic under activation; in CHO cells no difference in the SCE frequency compared to the control was observed with activation. This independence of NCDE mutagenic activity from metabolic activation in mammalian assay systems is in agreement with that found by Whong *et al.* (1983) in the *Salmonella*/microsome assay system.

The nitrosation process is important for establishing the genotoxicity of coal dust in mammalian cells, as indicated by Whong *et al.* (1983) in the *Salmonella* assay system. $NaNO_2$ did not induce SCEs in human lymphocytes. Nonnitrosated coal dust extract did produce an elevated SCE frequency in one of the two human subjects, but not in the other. This occurred only at the highest treatment dose,

TABLE 3
SISTER CHROMATID EXCHANGES INDUCED BY NITROSATED COAL DUST EXTRACT IN CHINESE HAMSTER OVARY CELLS

Treatment	Survival as % control		Number of SCEs[a]		SCEs per chromosome		SCEs/cell ± SE (21 chromosomes)	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Medium	100	100	442	501	0.43	0.48	9.0 ± 0.4	10.1 ± 0.4
DMSO (1%)	100	100	491	454	0.47	0.44	9.9 ± 0.4	9.2 ± 0.4
Mitomycin C (5.0 ng/ml)			1382		1.32		27.8 ± 0.7*	
Cyclophosphamide (1.5 μg/ml)				2204		2.12		44.5 ± 0.9*
NCDE								
33.3[b]	86	100	459	429	0.44	0.41	9.3 ± 0.4	8.7 ± 0.4
100.0	100	100	455	450	0.44	0.43	9.2 ± 0.4	9.1 ± 0.4
333.3	88	100	473	530	0.46	0.51	9.7 ± 0.4	10.7 ± 0.5*
1000.0	100	100	654	584	0.62	0.56	13.1 ± 0.5*	11.9 ± 0.5*
3333.0	61	22	884	—[c]	0.86	—	18.0 ± 0.6*	—

[a] Total for 50 cells.
[b] nl/ml.
[c] No metaphase cells.
* Significantly greater than solvent control, $P < 0.001$.

and the response was much less than that seen with NCDE. It is apparent that differences between individuals may be important in determining genotoxicity of some compounds. Blood samples from additional people are needed to determine the extent and significance of these individual differences. $NaNO_2$ and nonnitrosated coal dust alone did not induce chromosome aberrations.

Human lymphocyte chromosome aberration frequencies were also used to de-

TABLE 4
RESULTS OF THE MICRONUCLEUS TEST WITH NITROSATED COAL DUST EXTRACT IN MICE

Treatment	Dose[a]	Micronucleated cells per 1000 polychromatic erythrocytes (Mean ± SD)	Mean frequency[b]
DMSO[c]	15	1.60 ± 1.14	576
Cyclophosphamide[d]	15	25.40 ± 8.65	676
NCDE	5	3.00 ± 1.87	849
	7	3.00 ± 1.22	807
	10	2.00 ± 0.71	742
	15	1.40 ± 0.89	475
Coal dust extract	5	3.60 ± 1.67	602
$NaNO_2$[e]	15	1.00 ± 0.71	659

[a] ml/kg body weight, 5 animals per treatment group.
[b] Mean number of polychromatic cells per 1000 normochromatic cells.
[c] 1:3 (v/v) in sterile distilled water.
[d] 2.67 mg/ml.
[e] 7.5 mg/ml.

TABLE 5
INDUCTION OF GENE MUTATION BY NITROSATED COAL DUST EXTRACT IN MOUSE LYMPHOMA CELLS

Treatment	Cloning efficiency (% control)	Total mutant colonies	Total viable colonies ($\times 10^4$)	Mutant frequency ($\times 10^6$)
Solvent control	100.0	45.5[b]	272.5[b]	16.7
Untreated control	73.4	39.0	200.0	19.5
Ethylmethanesulfonate 0.5 μl/ml	35.6	465.0	97.0	479.4
NCDE				
125.0[a]	63.9	47.0	174.0	27.0
250.0	76.0	41.0	207.0	19.8
500.0	66.1	51.0	180.0	28.3
1000.0	103.5	118.0	282.0	41.8

[a] nl/ml.
[b] Mean of two determinations.

termine if the genetic activity of NCDE was restricted to either mitotic or nonmitotic cells. Testing was conducted during G_0 (the nonmitotic phase in which human lymphocytes normally reside) and S and G_2 (DNA synthesis and premitotic gap). NCDE was genetically active in each cell phase tested, with the G_0 phase being perhaps slightly more sensitive. This is indicated at the highest dose of NCDE by the greater total aberration frequency and the reduced number of metaphases available for scoring. The *in vitro* results indicate that NCDE is genetically active even in cells that are not normally mitotic; the situation *in vivo* may be the same.

The results of these assays are consistent with each other, with the exception of the *in vivo* micronucleus test. The negative results of the micronucleus test may be due to one or more of the following reasons: (1) the concentration was too low, (2) mutagenic compounds were deactivated in the animal body, (3) mutagenic compounds did not reach bone marrow cells, and (4) NCDE does not cause genetic damage detectable by the micronucleus assay system.

The situation in coal miners is different from the animal studies; the resulting neoplasms in miners are gastric (Matolo *et al.*, 1972; Rockette, 1977; Stocks, 1962) and exposure occurs over many years. Because the nitrosation reaction is postulated to occur in the stomach, this organ is the first to be exposed to the nitrosated coal dust chemicals. Since metabolic activation reduces the genotoxicity of NCDE, the half-life of the toxic compounds could be much shorter *in vivo* than if activation was not necessary. This decreased half-life would mean that only those tissues exposed to NCDE prior to metabolic conversion would be at a substantial genotoxic risk. This appears to be in agreement with the observation that coal miners have an increased rate of gastric cancer, but not neoplasms of other organs or tissues. It has to be noted, however, that the formation of mutagens by nitrosation of coal dust extract observed in the *in vitro* experiments may not occur *in vivo* in the stomach or may not be carcinogenic to humans.

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REFERENCES

Ames, B. N., McCann, J., and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat. Res.* **31,** 347–363.

Clive, D, and Spector, J. F. S. (1975). Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutat. Res.* **31,** 17–29.

Goto, K., Maeda, S., Kano, Y., and Sugiyama, T. (1978). Factors involved in differential Giemsa-staining of sister chromatids. *Chromosoma* **66,** 351–359.

Matolo, N. M., Klauber, M. R., Gorishek, W. M., and Dixon, J. A. (1972). High incidence of gastric carcinoma in a coal mining region. *Cancer* **29,** 733–737.

Perry, P., and Wolff, S. (1974). New Giemsa method for the differential staining of sister chromatids. *Nature (London)* **251,** 156–158.

Preston, R. J., Au, W., Bender, M. A., Brewen, J. G., Carrano, A. V., Heddle, J. A., McFee, A. F., Wolff, S., and Wassom, J. S. (1981). Mammalian *in vivo* and *in vitro* cytogenetic assays: A report of the U.S. EPA's Gene-tox Program. *Mutat. Res.* **87,** 143–188.

Rockette, H. E. (1977). Cause specific mortality of coal miners. *J. Occup. Med.* **19,** 795–801.

Schalm, D. W., Jain, N. C., and Carrol, E. J. (1975). "Veterinary Hematology," 3rd ed., p. 29. Lea & Febiger, Philadelphia.

Snedecor, G. W., and Cochran, W. G. (1968). "Statistical Methods," 6th ed., pp. 324–325. Iowa State Univ. Press, Ames.

Stocks, P. (1962). On the death rates from cancer of the stomach and respiratory diseases in 1940–53 among coal miners and other male residents in countries of England and Wales. *Brit. J. Cancer* **16,** 592–598.

Terasima, T., and Tolmach, L. J. (1961). Changes in X-ray sensitivity of Hela cells during the division cycle. *Nature (London)* **190,** 1210–1211.

Whong, W-Z., Long, R., Ames, R. G., and Ong, T. (1983). Role of nitrosation in the mutagenic activity of coal dust: A postulation for gastric carcinogenesis in coal miners. *Environ. Res.* **32,** 298–304.