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Single-strand breaks in deoxyribonucleic acid in fire fighters accidentally exposed to *o*-nitroanisole and other chemicals

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Objectives The aim of the study was to detect single-strand breaks in deoxyribonucleic acid (DNA) in mononuclear blood cells of fire fighters exposed to *o*-nitroanisole and other substances released into the environment during an accident in a chemical plant.

Methods The level of DNA single-strand breaks in mononuclear blood cells was detected by alkaline elution. The results were compared for 16 fire fighters who worked in a contaminated area for about 8 h and two reference groups (one of fire fighters who had not worked in the contaminated area, group I, and one of persons without any apparent occupational exposure to genotoxic substances, group II).

Results The mean normalized elution rate (nER) 19 d after the accident was slightly but statistically significantly ($P < 0.05$) higher for the exposed fire fighters [mean 1.48 ± 95% confidence interval (95% CI) 0.21] than for reference group I (mean 1.21 ± 95% CI 0.21) or reference group II (mean 1.17 ± 95% CI 0.18). No statistically significant difference was found between reference groups I and II. Another analysis was performed three months after the first. The level of DNA single-strand breaks (mean nER 1.12 ± 95% CI 0.11) was no longer increased in comparison with the levels of the reference groups.

Conclusions DNA single-strand breaks were increased in fire fighters exposed to *o*-nitroanisole and other substances. In comparison with the extent of DNA strand breaks found in other occupational groups the increase was only moderate. The observed decrease in DNA single-strand breaks to the reference level in exposed fire fighters three months later suggests a DNA repair mechanism for DNA single-strand breaks caused by *o*-nitroanisole.

Key terms acute toxicity, biomonitoring, DNA single-strand breaks, fire fighters, human mononuclear blood cells, *o*-nitroanisole.

During an accident in a chemical factory in Germany (22 February 1993) about 10 t of a vaporous mixture of substances was released from a methoxylation plant. The solid parts of the released vapor (3.5 t) precipitated in the area of the plant and in a suburb across the river Main (table 1). In the breathing air of this suburb, *o*-nitroanisole concentrations of up to 18 $\mu g \cdot m^{-3}$ were detected. In one street of the contaminated suburb 180—200 mg of *o*-nitroanisole precipitated per square meter of land. In a more distant street at the border of the contaminated area 5—10 mg of *o*-nitroanisole was detected per square meter of land. During the next few days this precipitate was removed mechanically with brushes, high-pressure cleaning machines, and so forth by workers from the chemical plant and by fire fighters. The fire fighters did not use protective masks or protective clothing at the beginning of their work in the contaminated area, because significant exposure levels were not expected. *o*-Nitroanisole, the most important substance emitted during the accident, is known to be mutagenic in the Ames test (1) and has caused cancer in experimental animals (2). Exposure to *o*-nitroanisole can occur through inhalation, penetration of the skin (3), and ingestion. Several other chemicals released during the accident, such as *o*-anisidine and *o*-chloronitrobenzene, are also considered genotoxic (4—8).

In this study we used a modified alkaline elution method to investigate DNA single-strand breaks in mononuclear blood cells of fire fighters who were working in the contaminated area. By alkaline elution the genotoxic

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effect of all substances inducing DNA single-strand breaks or adducts which render the DNA alkali labile can be detected. Therefore, it is not necessary to adapt the method for individual substances, and also the effect of unknown genotoxins can be determined. Therefore the alkaline elution method is suitable for biomonitoring the effect of mixtures of various chemicals. DNA single-strand breaks or alkali labile sites are known to be induced by the majority of genotoxic animal carcinogens. Sina et al (8) used the alkaline elution assay for detecting DNA single-strand breaks in rat hepatocytes and reported that the assay had a high potential to identify genotoxic carcinogens. In humans, DNA single-strand breaks have previously been used as an indicator of genotoxic exposure in a few cases. Walles et al (9) and Mäki-Paakkanen et al (10) detected DNA single-strand breaks to biomonitor human exposure to styrene using the DNA unwinding method or the alkaline elution technique (11). Recently we described a modified alkaline elution procedure for biomonitoring DNA single-strand breaks in mononuclear blood cells of humans exposed to genotoxic substances (12—14). In the present study we have compared the number of DNA single-strand breaks in fire fighters accidently exposed to *o*-nitroanisole and several other chemicals with those of a matched group of fire fighters who were not in the contaminated area (reference group I) and with other individuals without any apparent occupational exposure to genotoxic substances (reference group II).

Subjects and methods

Subjects

The exposed fire fighters were healthy men who had been exposed to a mixture of substances (table 1) released during an accident in a chemical plant. They had worked in the contaminated area for about 8 h. One fire fighter had been exposed for 40 h. Detailed information on the duties carried out in the contaminated area is given in table 2. During the exposure, none of the fire fighters had worn protective clothing, nor had other safety provisions been applied.

Reference group I consisted of unexposed fire fighters who had not worked in the contaminated area. They were matched with the exposed fire fighters for age, alcohol consumption, town of residence, and the number of cigarettes smoked in the group of smokers. The exposed fire fighters were still in training and had not yet been involved in fire fighting, with the exception of subject F2. The firefighting activity of this subject and of the unexposed fire fighters (reference group I) was relatively low (less than two fires per month). Another group of referents (group II) was constructed of male students and office employees without any apparent occupational exposure to genotoxic substances.

An interviewer-administered questionnaire was used to collect data from each subject prior to the collection of the blood samples. The questionnaire included information on cigarette smoking (number of cigarettes currently smoked per day, lifetime smoking history), age, health history, intake of alcohol and drugs, exposure to putative carcinogens or ionizing radiation, occupation, and length of occupation and occupational exposure. The fire fighters were asked to give the time they had worked in the contaminated area and the duties they had carried out, use of protective clothing, and complaints related to their exposure in the contaminated area. The age, smoking habits, and occupation of the referents and fire fighters, as well as the complaints of the fire fighters in relation to exposure and their duty carried out in the contaminated region are summarized in tables 2—4. The exposed fire fighters and reference groups I and II were similar with respect to the distributions of age and the number of cigarettes smoked in the group of smokers. Alcohol consumption was similar for the exposed [4.2 (SD 2.2) g of alcohol a day] and unexposed [5.0 (SD 2.5) g a day] fire fighters but higher for reference group II [10.9 (SD 4.5) g a day]. None of the subjects used drugs or had apparently been exposed to genotoxic substances (besides the exposure studied) or ionizing radiation.

Table 1. Substances precipitated during the accident in the chemical plant, given in mass percentage. A total of 3.5 t precipitated.

Substance	Percentage
o-Nitroanisole	27.8
Sodium formate	14.0
Sodium chloride	11.6
Water	12.2
2,2'-Dichloroazoxybenzene	7.8
2,2'-Dichloroazobenzene	4.9
Dimethoxyazoxybenzene	4.2
o-Nitrophenol	3.5
Chloromethoxyazoxybenzene	2.5
o-Chloroaniline	1.5
o-Chloronitrobenzene	1.4
Methanol	1.1
Sodium carbonate	0.9
o-Anisidine	0.7
2,2'-Chloronitrodiphenylamine	0.7
Chloromethoxyazobenzene	0.6
Nitrobenzene	0.3
Chloroazoxybenzene	0.3
Chlorohydroxyazobenzene	0.3
Substituted biphenyl ($C_{12}H_9O_3N_2Cl$)	0.3
Substituted diphenylamine ($C_{12}H_9N_2Cl$)	0.3
Sodium nitrite	0.3
Substituted diphenylamine ($C_{12}H_{11}ON_2Cl$)	0.2
o-Chloroanisole	0.1
Phenazine	0.1
Sodium hydroxide	0.1
Dimethoxyazobenzene	0.1
Inorganic contaminants (rust, salts)	about 2.0

Table 2. Age, smoking habits, normalized elution rates, individual duty carried out in the contaminated region, and exposure-related complaints of the fire fighters exposed to *o*-nitroanisole and other chemicals. The measurements were performed 19 d after the exposure (first analysis) and 88 d later (second analysis). The time of exposure was about 8 h for all of the fire fighters except F2, who was exposed for 40 h. None of the fire fighters wore protective clothing, except F16 who wore gloves. (NT=not tested)

Code number of fire fighter	Age (years)	Cigarettes per day	Normalized elution rates		Duty carried out	Complaints related to exposure
			First analysis	Second analysis		
F1	24	—	1.31	1.31	Reconnaissance of the contaminated region	Skin rash around mouth and nose for one day
F2	46	—	2.26	0.90	Reconnaissance	Nose bleeding for one day, chapped lip
F3	30	—	2.03	1.10	Reconnaissance, distribution of leaflets	Dry cough for two days
F4	23	—	1.22	0.67	Reconnaissance, distribution of leaflets	Headache for one day, burning of the eyes for one day
F5	31	15	0.80	1.13	Reconnaissance	Nose bleeding for one day, headache for seven days
F6	32	20	1.34	1.02	Distribution of leaflets	None
F7	30	—	2.13	1.14	Reconnaissance	Burning of the eyes for one day
F8	30	15	1.35	1.11	Truck driver, loading of contaminated material	Nose bleeding, skin rash on the neck, headache for one day, influenzal infection after exposure
F9	29	8	NT	0.96	Commanding officer of fire fighting unit	Irritation of the mouth mucous membrane, burning of the eyes for one day
F10	28	—	1.31	0.99	Reconnaissance	None
F11	28	10	NT	1.26	Reconnaissance	Irritation of the upper respiratory tract for one day
F12	25	—	1.39	1.32	Reconnaissance	None
F13	26	15	1.17	0.94	Reconnaissance	Irritation of the upper respiratory tract for one day
F14	22	—	1.59	1.67	Reconnaissance	None
F15	24	—	1.47	1.30	Reconnaissance	Headache for one day
F16	33	—	1.29	1.03	Reconnaissance, cleaning of the railing of a bridge for one hour	Skin rash on the neck and back for one day, irritation of the upper respiratory tract for one day
Complete group						
Mean value	28.8	5.2	1.48	1.12		
95% confidence interval	2.8	3.6	0.21	0.11		
Nonsmokers						
Mean value	28.5	—	1.60	1.14		
95% confidence interval	4.4	—	0.24	0.17		
Smokers						
Mean value	29.3	13.8	1.17	1.07		
95% confidence interval	1.7	3.4	0.25	0.10		

Alkaline elution

Blood samples for the determination of DNA single-strand breaks were collected from the subjects 19 d after the exposure and also three months later. Venous blood, 20 ml per person, was taken into heparinized tubes, transported to the laboratory on ice, and processed within 3 h. Mononuclear blood cells were isolated by metrizoate-Ficoll centrifugation according to Boyum (15). The alka-

Table 3. Smoking habits, age, and normalized elution rates of the unexposed fire fighters (reference group I).

Code number of fire fighter	Age (years)	Cigarettes per day	Normalized elution rates
FC1	26	15	1.08
FC2	24	—	1.40
FC3	34	—	2.17
FC4	23	—	0.49
FC5	24	—	1.32
FC6	29	—	1.11
FC7	27	—	1.56
FC8	26	—	0.99
FC9	29	20	1.31
FC10	27	—	1.20
FC11	24	—	2.28
FC12	29	—	1.02
FC13	26	18	0.43
FC14	28	—	1.14
FC15	21	13	0.88
FC16	27	15	1.15
FC17	26	—	0.89
FC18	26	—	1.36
FC19	23	—	1.14
Complete group			
Mean value	26.1	4.3	1.21
95% confidence interval	1.3	3.4	0.21
Nonsmokers			
Mean value	26.4	—	1.29
95% confidence interval	1.6	—	0.25
Smokers			
Mean value	25.8	16.2	0.97
95% confidence interval	2.6	2.4	0.30

Table 4. Age, smoking habits, occupation, and normalized elution rates of persons without any apparent genotoxic occupational exposure (reference group II).

Code number of referent	Age (years)	Cigarettes per day	Occupation	Normalized elution rate
1	24	20	Student	1.00
2	26	20	Student	2.11
3	25	15	Student	0.94
4	25	—	Student	0.82
5	29	18	Student	0.91
6	40	—	Office clerk	0.95
7	26	20	Student	1.23
8	22	10	Student	0.69
9	26	20	Student	1.36
10	26	—	Student	1.55
11	42	—	Office clerk	1.15
12	28	—	Student	1.27
13	28	15	Student	0.93
14	26	—	Student	1.72
15	23	—	Student	1.84
16	52	—	Nurse	1.20
17	28	—	Nurse	0.75
18	22	—	Student	0.55
19	22	—	Student	0.37
20	50	20	Cleaner	0.22
21	28	20	Student	1.42
22	27	—	Student	2.10
23	25	—	Student	1.04
24	28	—	Student	1.18
25	28	5	Student	1.61
26	24	—	Student	1.26
27	27	—	Student	0.78
28	25	3	Student	1.68
Complete group				
Mean value	28.6	6.6		1.17
95% confidence interval	2.9	3.3		0.18
Nonsmokers				
Mean value	29.1	—		1.16
95% confidence interval	4.1	—		0.23
Smokers				
Mean value	28.1	15.5		1.18
95% confidence interval	4.1	3.5		0.28

line elution method of Kohn et al (16) was employed with some modifications. A suspension of two million mononuclear blood cells in 1 ml of cold phosphate buffered saline was poured onto a polycarbonate filter (Nucleopore, Tübingen, Germany, 25 mm diameter, 2 μm pore size). The cells were lysed with 3 ml of a solution of 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, and 2 M sodium chloride (NaCl) (adjusted to pH 10 with sodium hydroxide) for 60 min. The lysed cells were washed with 4.5 ml of 10 mM EDTA (adjusted to pH 10 with sodium hydroxide) using a flow rate of 3 ml · h^{-1} for 90 min. Before the sampling of the eluted DNA was started, one fraction equal to the volume of the tube (0.25 ml) was discarded. The elution was performed at 4°C in the dark using a 5 M NaCl, 2 mM EDTA solution adjusted to pH 12.6 with sodium hydroxide. With a pump speed of 1.5 ml · h^{-1}, the eluate was collected over a period of 10 h. The filters were removed and sonicated in a 15-ml elution solution for 2×15 min (filter fraction). Quantification of the DNA was performed as described earlier (13). For standardization, DNA of untreated V79 cells, a Chinese hamster lung fibroblast cell line, was eluted simultaneously in every run on separate filters. V79 cells were cultured as described earlier (13). DNA concentrations of the eluted fractions, and of the filter fractions, were determined in triplicate. The elution rate was calculated as $-(\log_{10}R) \cdot 0.1\ h^{-1}$, in which R represents the fraction of DNA remaining on the filter after 10 h of elution. The normalized elution rate is expressed as the ratio of the elution rate of test cell DNA to the elution rate of V79 DNA. The DNA of the peripheral mononuclear blood cells, as well as the DNA of the V79 cells, was eluted on four separate filters each, and the mean values of these four elution runs were calculated. Samples from the exposed fire fighters, reference group I, and reference group II were analyzed simultaneously, and samples from a

constant number of persons from each of the three groups were analyzed per elution run.

The U-test, according to Wilcoxon, Whitney, and Mann (two-sided) was applied to evaluate the statistical significance of differences in unpaired data, and Wilcoxon's signed rank test was used to evaluate paired data. The 95% confidence intervals (95% CI) were calculated as $1.96 \cdot SD/n^{0.5}$, where SD represents the standard deviation and n the number of persons. The mean values ± 95% confidence intervals are given. The statistical significance of the correlation coefficients of the linear regression was evaluated with the use of the t-statistic.

Results

The mean normalized elution rate of the fire fighters working in the contaminated area was 1.48 (± 95% CI 0.21) in the first measurement, which was performed 19 d after the exposure (table 2). For the unexposed fire fighters (reference group I) living in the same town a mean normalized elution rate of 1.21 (± 95% CI 0.21) was obtained (table 3). The difference between the exposed fire fighters and reference group I was statistically significant ($P < 0.05$, U-test). The mean normalized elution rate of reference group II was 1.17 (± 95% CI 0.18) (table 4), and it did not differ statistically significantly from that of reference group I, but was statistically significantly smaller than the mean normalized elution rate of fire fighters exposed to *o*-nitroanisole ($P < 0.05$, U-test).

Another analysis of DNA single-strand breaks was performed for the same exposed fire fighters 88 d after the first analysis (table 2). The mean normalized elution rate in this investigation was 1.12 (± 95% CI 0.11), which was similar to that obtained for the reference groups and was statistically significantly smaller than the mean elution rate in the first analysis ($P < 0.01$, Wilcoxon's signed rank test).

In several other groups occupationally exposed to genotoxic substances, we detected a higher increase in DNA single-strand breaks in nonsmokers than in smokers (12, 14). Therefore all persons tested in this study were analyzed according to their smoking habits (tables 2—4). No statistically significant differences could be detected between the smokers and nonsmokers in reference groups I and II. In the first analysis, the mean normalized elution rate of the nonsmoking fire fighters who had worked in the contaminated area was significantly higher [1.60 (± 95% CI 0.24] compared with that of nonsmoking persons from reference group I [1.29 (± 95% CI 0.25)] and reference group II [1.16 (± 95% CI 0.23)] ($P<0.05$, U-test). No statistically significant difference in the normalized elution rates could be found between the smoking exposed fire fighters [1.17 (± 95% CI 0.25)] and the smokers from reference groups I [0.97 (± 95% CI 0.30)] and II (1.18 ± 95% CI 0.28). However, it should be mentioned that the number of individuals was very small in these subgroups. The mean normalized elution rate was 27% higher in the total group of smoking and nonsmoking fire fighters when compared with that of reference group II and 38% higher if only nonsmokers were considered.

No statistically significant correlations (t-statistics of correlation coefficients) were found between the extent of DNA damage and age or alcohol consumption for the individuals in reference groups I and II and for the exposed fire fighters.

An analysis of the elution rates of the exposed fire fighters with respect to the length of exposure could not be performed because almost all of the fire fighters were exposed for 8 h with the exception of one person who was exposed for 40 h. Nevertheless, it is striking that this particular fire fighter (F2) also showed the highest mean normalized elution rate (2.26) within the group of fire fighters exposed to *o*-nitroanisole.

Twelve of the 16 fire fighters complained of symptoms occurring after their work in the contaminated region (table 2). Skin irritation of the neck or around the mouth was reported by three persons. Three fire fighters complained of burning of the eyes, and four noted an irritation of the upper respiratory tract. Three fire fighters reported nose bleeding and four complained about headache.

Discussion

An increase in DNA single-strand breaks of 22% was detected in fire fighters exposed to *o*-nitroanisole compared with unexposed fire fighters matched for age, cigarette smoking, and alcohol consumption. Liou et al (17) detected an increased number of benzo[a]pyrene diol epoxide-DNA adducts in fire fighters. Therefore an increased number of DNA single-strand breaks in mononuclear blood cells of fire fighters could also be expected, possibly due to skin contact or fumes inhaled during fire fighting. In this study, however, similar numbers of DNA single-strand breaks were detected in fire fighters not exposed to *o*-nitroanisole (reference group I) and persons without any apparent occupational exposure to genotoxic substances (reference group II).

In the second analysis, which was performed 88 d after the first, the number of DNA single-strand breaks was no longer higher in the exposed fire fighters than in the unexposed fire fighters or the persons from reference group II. Possible mechanisms for the observed decrease in DNA single-strand breaks could be DNA repair or the

turnover of mononuclear blood cells. Mononuclear blood cells of healthy donors consist of T lymphocytes (about 81%), B lymphocytes (about 3%), and monocytes (about 16%). T lymphocytes consist of a larger long-living subpopulation (at least 90%) and of a smaller subpopulation (about 10%) with half-times of 300—1000 d and less than two weeks, respectively. The majority of B lymphocytes is short-lived (half-time shorter than two weeks), and monocytes leave the vascular system with a mean half-time of 8.4 h (18). With the use of these numbers, it can be calculated that about 66% of the mononuclear blood cells present in the first analysis were also present in the second analysis 88 d later. According to these data the disappearance of DNA single-strand breaks during a period of 88 d was not likely to be due exclusively to the turnover of mononuclear cells. Therefore the decrease in DNA single-strand breaks seems to have been caused by a slow DNA repair mechanism, which could not remove all of the single-strand breaks induced by *o*-nitroanisole within the 19 d, but was able to reduce DNA single-strand breaks to reference levels 88 d later.

Little is known about human genotoxic effects of the substances released during the accident. *o*-Nitroanisole, which represented the main fraction of substances released (25.3%), was mutagenic in the Ames test (1) and caused cancer in animals (2). *o*-Chloronitrobenzene, which represented 1.1% of the substances released, was mutagenic in the presence of rat liver microsomes as the metabolic activating system (6), and caused tumors in rats (7). *o*-Anisidine, representing 0.6% of the substances released, caused DNA single-strand breaks in mouse lymphoma cells in the presence of rat liver microsomes (4), and also caused bladder cancer in rats and mice (5).

In earlier studies, we analyzed DNA single-strand breaks in mononuclear blood cells of persons occupationally exposed to putative carcinogens. For nonsmoking workers, compared with referents, the increase in DNA single-strand breaks was found to be 60% for painters, 70% for workers exposed to ethylene oxide, and 69% for metal workers exposed to cutting fluids (12, 14). In smokers consuming more than 10 cigarettes per day, 13% more single-strand breaks could be detected than in nonsmokers (12). In comparison with the results of earlier studies, the mean normalized elution rate of the fire fighters exposed to *o*-nitroanisole was increased to a relatively low extent (38% in the nonsmoking fire fighters) in comparison with that of the nonsmoking referents. Furthermore, individuals from the aforementioned occupational groups are usually exposed to the substances continuously during their occupational life, whereas the fire fighters were exposed to the accidentally emitted *o*-nitroanisole and other compounds of the mixture for a short period only.

The determination of an equivalent concentration of a well-known genotoxic substance such as ethylene oxide could help one assess the risk for the exposed fire fighters. However, this kind of risk assessment is indirect because it is not known whether DNA damage caused by ethylene oxide or *o*-nitroanisole has similar biological relevance. The detection of DNA single-strand breaks in mononuclear blood cells of persons exposed to ethylene oxide revealed an increase in normalized elution rates of 0.19 if the concentration of ethylene oxide was elevated by 1 $mg \cdot m^{-3}$ (4-h time-weighted average) (14). Therefore an increase in normalized elution rates by 0.27, calculated for the fire fighters compared with persons without any apparent occupational exposure to genotoxic substances, would be equivalent to exposure to an ethylene oxide concentration of 1.4 $mg \cdot m^{-3}$. Human exposure to ethylene oxide concentrations of almost 2 $mg \cdot m^{-3}$ (occupational exposure limit) may be tolerated during their complete occupational life, whereas the fire fighters were exposed to the accidentally emitted substances for a short period of time. Therefore we estimate only a minor genotoxic hazard for the fire fighters due to their service in the accidentally contaminated area, compared with the genotoxic hazard of other occupational groups. However, these considerations have to be treated with caution since, at present, it is not known whether DNA single-strand breaks or alkali labile sites caused by ethylene oxide or *o*-nitroanisole have similar biological consequences.

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