Carcinogenesis vol.24 no.1 pp.63–73, 2003

Occupational exposure to heavy metals: DNA damage induction and DNA repair inhibition prove co-exposures to cadmium, cobalt and lead as more dangerous than hitherto expected

Jan G.Hengstler[1,7], Ulrich Bolm-Audorff[4], Andreas Faldum[5], Kai Janssen, Michael Reifenrath[1], Walter Götte[1], Detlev Jung[2], Otfried Mayer-Popken[2], Jürgen Fuchs[3], Susanne Gebhard[1], Heinz Günter Bienfait[4], Kirsten Schlink[1], Cornelia Dietrich[1], Dagmar Faust[1], Bernd Epe[6] and Franz Oesch[1]

[1]Institute of Toxicology, Obere Zahlbacher Str. 67, 55131 Mainz, [2]Institute of Occupational Medicine, Obere Zahlbacher Str. 67, 55131 Mainz, [3]Institute of Environmental Toxicology, Werrastrasse 5, 99834 Gerstungen, [4]Ministry of Labour Inspection, Occupational Health Division, Dostojewskistr. 4, 65187 Wiesbaden, [5]Institute for Medical Biostatistics, Epidemiology and Informatics, University of Mainz, 55101 Mainz and [6]Institute of Pharmacy, University of Mainz, 55131 Mainz, Germany

[7]To whom correspondence should be addressed
Email: hengstle@mail.Uni-Mainz.de

Co-exposure to cadmium, cobalt, lead and other heavy metals occurs in many occupational settings, such as pigment and batteries production, galvanization and recycling of electric tools. However, little is known about interactions between several heavy metals. In the present study we determined DNA single strand break (DNA-SSB) induction and repair capacity for 8-oxoguanine in mononuclear blood cells of 78 individuals co-exposed to cadmium (range of concentrations in air: 0.05–138.00 μg/m^3), cobalt (range: 0–10 μg/m^3) and lead (range: 0–125 μg/m^3). Exposure to heavy metals was determined in air, blood and urine. Non-parametric correlation analysis showed a correlation between cadmium concentrations in air with DNA-SSB ($P = 0.001$, R = 0.371). Surprisingly, cobalt air concentrations correlated even better ($P < 0.001$, R = 0.401), whereas lead did not correlate with DNA-SSB. Logistic regression analysis including 11 possible parameters of influence resulted in a model showing that cobalt in air, cadmium in air, cadmium in blood and lead in blood influence the level of DNA-SSB. The positive result with cobalt was surprising, since exposure levels were much lower compared with the TRK-value of 100 μg/m^3. To examine, whether the positive result with cobalt is stable, we applied several logistic regression models with two blocks, where all factors except cobalt were considered preferentially. All strategies resulted in the model described above. Logistic regression analysis considering also all possible interactions between the relevant parameters of influence finally resulted in the following model: Odds ratio = $1.286^{\text{Co in air}} \times 1.040^{\text{Cd in air}} \times 3.111^{\text{Cd in blood}} \times 0.861^{\text{Pb in air}} \times 1.023^{\text{Co in air} \times \text{Pb in air}}$. This model correctly predicts an increased level of DNA-SSB in 91% of the subjects in our study. One conclusion from this model is the existence of more than multiplicative effects for co-exposures of cadmium, cobalt and lead. For instance increasing lead air concentrations from 1.6 to 50 μg/m^3 in the presence of constant exposures to cobalt and cadmium (8 μg/m^3 and 3.8 μg/m^3) leads to an almost 5-fold increase in the odds ratio, although lead alone does not increase DNA-SSB. The mechanism behind these interactions might be repair inhibition of oxidative DNA damage, since a decrease in repair capacity will increase susceptibility to reactive oxygen species generated by cadmium or cobalt. Indeed, repair of 8-oxoguanine decreased with increasing exposures and inversely correlated with the level of DNA-SSB ($P = 0.001$, R = –0.427). Protein expression patterns of individuals exposed to cobalt concentrations of ~10 μg/m^3 were compared with those of unexposed individuals using two-dimensional gel electrophoresis. Qualitative and apparent quantitative alterations in protein expression were selective and certainly occurred in <0.1% of all proteins. In conclusion, the hazard due to cobalt exposure – that has been classified only as IIB by the IARC – seems to be underestimated, especially when individuals are co-exposed to cadmium or lead. Co-exposure may cause genotoxic effects, even if the concentrations of individual heavy metals do not exceed TRK-values.

Introduction

The International Agency for Research on Cancer classified cadmium as a human carcinogen (1). According to early reports an excess of prostate cancer was found among workers with high exposure to cadmium oxide at nickel–cadmium battery factories (2–5), but epidemiologic studies have not consistently supported the early reports of excess of prostate cancer in cadmium-exposed workers (6–8). The evaluation of the IARC is based mainly on the results from studies of cadmium smelter workers (9). In the latter study excess mortality from lung cancer was observed for the cadmium-exposed workers (SMR = 149; 95% confidence interval = 95, 222) (9). Risk increased with estimates of increasing cadmium exposure (9). The lifetime excess lung cancer risk for cadmium fumes of 100 μg/m^3 was estimated to be ~50–111 lung cancer deaths per 1000 workers exposed to cadmium for 45 years (9). Similarly in a study of a cohort of Swedish battery workers exposed to cadmium oxide and nickel hydroxide an increased risk for lung cancer, cancer of the nose and nasal sinuses was observed, although a differentiation between the effects of cadmium and nickel was not possible in this study (10). Similar to human carcinogenicity, cancer of the lung has also been induced in rats by respiratory exposure to cadmium compounds (1).

Bacterial mutagenicity tests with cadmium salts (Cd^{2+}) were mostly negative (11–14) and mutagenicity in mammalian cells was only weakly positive (15,16). Chromosomal aberrations in mammalian cells *in vitro* were positive only at highly cytotoxic concentrations, suggesting that induction of chromosomal aberrations was due rather to toxicity than direct DNA interaction (17). Clear-cut positive results were obtained for

Abbreviations: AAS, atomic absorption spectrophotometry; DNA-SSB, single strand break(s).

micronuclei in V79 Chinese hamster cells *in vitro* (18) and for DNA single strand break (DNA-SSB) induction in V79 Chinese hamster cells *in vitro* (19,17) as well as in the rat lung, kidney, brain and liver after application of cadmium (Cd^{2+}) *in vivo* (20,21). Thus both micronuclei as well as DNA-SSB seem to be adequate endpoints for genotoxic effect-biomonitoring in cadmium exposed workers. In the present study we preferred determination of DNA-SSB, since determination by alkaline elution is less time consuming and almost completely automatized (22,23).

The principal mechanisms of cadmium genotoxicity, mutagenicity and carcinogenicity are: (i) generation of reactive oxygen species (17,24–26); (ii) inhibition of DNA repair (14,17,27–31); (iii) depletion of glutathione (17,32); and (iv) possibly also suppression of apoptosis (33). Inhibition of DNA repair, namely nucleotide excision repair (27–29), base excision repair (30), DNA-SSB rejoining (31) and O^6-methylguanine-DNA-methyltransferase (14,17) may be of high relevance for human carcinogenesis, since DNA repair inhibition by cadmium occurs already at very low (for practical exposure scenarios) concentrations. For instance nucleotide excision repair was already inhibited at 0.5 μM cadmium (Cd^{2+}), whereas direct DNA damaging effects are usually observed at higher concentrations. Inhibition of DNA repair may also explain the observation that cadmium, although non-mutagenic in bacteria, enhances mutagenicity of, for instance, methyl nitrosourea (13,14), and although only weakly mutagenic in mammalian cells itself, strongly enhances UV-induced mutagenicity in mammalian cells (15).

World-wide ~15 000 tons of cadmium are produced per year. Cadmium is used mainly in nickel–cadmium batteries, in pigments, as a chemical stabilizer, in metal coatings, and as an alloy (5). Exposure to the general population occurs mainly by industrial contamination of topsoil via uptake into food plants and tobacco. However, human uptake from dietary sources is usually relatively small (1–3 μg cadmium/day). More problematic is occupational exposure, since cadmium levels of >1 mg/m^3 have been reported in the air of working places of cadmium production and refining industries, alloy production and battery manufacture (1). Because of the low excretion rate (biological half-life: 10–30 years), cadmium accumulates in the body, particularly in the kidneys, liver and bones.

A large number of individuals are occupationally exposed to cadmium in all industrial countries. Usually, workers are not exposed to cadmium as a single substance, but co-exposed to other heavy metals, especially cobalt, lead and nickel. Little is known about interactions between heavy metals during co-exposure in humans. In the present study we examined levels of DNA-SSB and repair activity of 8-oxoguanine in mononuclear blood cells of 78 workers occupationally exposed to cadmium, cobalt and lead.

Materials and methods

Subjects

The exposed individuals ($n = 78$, 16 women and 62 men) were employed in ten different facilities in Germany in Hessen. These facilities were selected because high air concentrations of cadmium were expected. The ten facilities were specialized in production of cadmium containing pigments (facility nos. 2,3,7), production of cadmium containing batteries (facility no. 9), galvanization (facility no. 4) and recycling of electric tools, especially television sets (facility nos. 1,5,6,8,10). An interviewer-administered questionnaire was used to collect data from each subject prior to the collection of the blood samples. The questionnaire included questions on cigarette smoking (number of cigarettes currently smoked per day and lifetime smoking history), daily intake of alcohol (special care was taken to differentiate between a daily ethanol consumption $>$ or <5 g), intake of pharmaceuticals, exposure to carcinogens or ionizing radiation, length of occupation, duties carried out, use and type of protective clothing, breathing masks, health status, complaints related to work, daily exposure time to cadmium substances, ethnicity, gender and age. None of the subjects had apparently been exposed to other genotoxic substances than heavy metals or to ionizing radiation. All individuals examined gave written consent to joining this study.

The following parameters were determined for all exposed individuals: cadmium, cobalt, nickel and lead concentrations in the air of the working place. Air was collected using personal samplers. Collection time for air was 6 h immediately before blood and urine samples were taken. Thus, air concentrations represent mean exposures during the 6 h before blood cells were taken for DNA-SSB analysis, whereas concentrations in blood and urine reflect longer periods of exposures of the recent past. Concentrations of heavy metals were determined by atomic absorption spectrophotometry (AAS). Concentrations of cadmium and lead in blood were also determined by AAS in heparinized venous blood after homogenization and precipitation of proteins. Cadmium and cobalt concentrations in urine were determined by AAS after acidification of the urine with 1 M HNO_3 (urine: HNO_3 = 1:3 vol/vol) and centrifugation to remove protein or particles. Urine samples were taken at the end of the working day. In addition, iron in serum and creatinine in urine were determined by routine techniques. Determination of heavy metal concentrations were performed at the Labour Inspection Laboratory in Wiesbaden, whereas DNA damage was measured at the Institute of Toxicology, Mainz. The data on heavy metal concentrations were kept secret by the Labour Inspection Laboratory in Wiesbaden, until the analysis of DNA damage had been finished and submitted to the Ministry of Work.

An additional control group ($n = 22$) without occupational exposure to heavy metals with a similar age distribution as the exposed individuals consisted of healthy office employees ($n = 7$), laboratory assistants ($n = 10$) and medical students ($n = 5$). None of the subjects from the control group used pharmaceuticals or had apparently been exposed to genotoxic substances or ionizing radiation. Subjects from the control group came from a similar geographical region (Hessen and Rheinland-Pfalz in mid-Germany) as the exposed individuals, but were not individually matched. Thus, the control group ($n = 22$) was not included into regression analysis but was used to dichotomize DNA single strand breaks into 'normal' and 'increased' levels. Logistic regression analysis and analysis of correlation was performed only within the 78 individuals occupationally exposed to heavy metals.

DNA single strand break analysis

Blood samples for determination of DNA-SSB were collected from the exposed individuals at the end of a working day. Venous blood, 20 ml per individual, was taken into heparinized tubes, transported to the laboratory on ice and processed within 3 h. Blood samples from the controls were taken at the same day as from the exposed individuals and were processed in parallel. Mononuclear blood cells were isolated by metrizoate-Ficoll centrifugation (22) and cryopreserved by a technique originally established for hepatocytes, but which is also suitable for cryopreservation of human mononuclear blood cells for alkaline elution analysis (34). The alkaline elution method (22,23) and trypan blue exclusion analysis (35) were performed as described previously. Normalized elution rates were calculated as a measure for the level of DNA single strand breaks (22).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described (36). Thirty μg protein were loaded on each gel.

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 software (SPSS, Chicago, IL). Quartiles, minimal and maximal values, are given for continuous variables. Non-parametric Spearman's rank correlation analysis and scatterplots were used to examine correlations between DNA-SSB and exposure to heavy metals and between different types of exposure. DNA-SSB were dichotomized into 'normal' and 'increased' by the one-sided 90% confidence interval of non-exposed subjects (control group). To identify relevant parameters of influence (model finding) a multivariable logistic regression model was used. Eleven possible parameters of influence were included: cadmium in air, cadmium in blood, cobalt in air, cobalt in urine, lead in air, lead in blood, iron in serum, age, gender, cigarette pack years, and alcohol consumption. Continuous parameters were taken into account by their original values and simultaneously as categorized variables. Categorization was carried out based on the quartiles of the respective continuous variable. To evaluate the influence of cobalt an additional logistic regression model with two blocks was applied. The first block contained all influential parameters without cobalt. The second block contained cobalt in order to adjust cobalt to all the parameters found to

Table I. Spearman's rank-correlation analysis: correlation of exposure to heavy metals and the extent of DNA-SSB in mononuclear blood cells. Coefficients of correlation (R) and two sided *P*-values are given

		DNA single strand breaks	Cadmium in air (μg/m^3)	Cadmium in blood (μg/l)	Cadmium in urine (μg/l)	Cadmium in urine normalized to creatinine (μg/g)	Cobalt in air (μg/m^3)	Cobalt in urine (μg/l)	Cobalt in urine normalized to creatinine (μg/g)	Lead in air (μg/m^3)	Lead in blood (μg/l)
DNA-SSB (normalized elution rates)	R	1.000	0.371	0.220	0.076	0.211	0.401	0.347	0.381	0.027	–0.057
	P-value	.	0.001	0.053	0.510	0.074	0.000	0.002	0.001	0.816	0.622
	n	78	75	78	77	0.73	76	76	73	76	78
Cadmium in air (μg/m^3)	R	0.371	1.000	0.043	0.002	–0.060	0.267	0.076	0.051	0.231	–0.315
	P-value	0.001	.	0.716	0.989	0.620	0.021	0.525	0.674	0.047	0.006
	n	75	75	75	74	70	75	73	70	75	75
Cadmium in blood (μg/l)	R	0.220	0.043	1.000	0.422	0.499	0.210	0.230	0.255	0.185	0.290
	P-value	0.053	0.716	.	0.000	0.000	0.069	0.046	0.030	0.110	0.010
	n	78	75	78	77	73	76	76	73	76	78
Cadmium in urine (μg/l)	R	0.076	0.002	0.422	1.000	0.760	0.092	0.234	0.158	0.294	0.286
	P-value	0.510	0.989	0.000	.	0.000	0.431	0.042	0.181	0.010	0.012
	n	77	74	77	77	73	75	76	73	75	77
Cadmium in urine normalized to creatinine (μg/g)	R	0.211	–0.060	0.499	0.760	1.000	0.223	0.167	0.229	0.186	0.314
	P-value	0.074	0.620	0.000	0.000	.	0.062	0.160	0.053	0.121	0.007
	n	73	70	73	73	73	71	72	72	71	73
Cobalt in air (μg/m^3)	R	0.401	0.267	0.210	0.092	0.223	1.000	0.453	0.504	0.368	0.460
	P-value	0.000	0.021	0.069	0.431	0.062	.	0.000	0.000	0.001	0.000
	n	76	75	76	75	71	76	74	71	76	76
Cobalt in urine (μg/l)	R	0.347	0.076	0.230	0.234	0.167	0.463	1.000	0.963	0.058	0.273
	P-value	0.002	0.525	0.046	0.042	0.160	0.000	.	0.000	0.622	0.017
	n	76	73	76	76	72	74	76	73	74	76
Cobalt in urine normalized to creatinine (μg/g)	R	0.381	0.051	0.255	0.158	0.229	0.504	0.963	1.000	0.010	0.260
	P-value	0.001	0.674	0.030	0.181	0.053	0.000	0.000	.	0.937	0.026
	n	73	70	73	73	72	71	73	73	71	73
Lead in air (μg/m^3)	R	0.027	0.231	0.185	0.294	0.186	0.368	0.058	0.010	1.000	0.417
	P-value	0.816	0.047	0.110	0.010	0.121	0.001	0.622	0.937	.	0.000
	n	76	75	76	75	71	76	74	71	76	76
Lead in blood (μg/l)	R	–0.057	–0.315	0.290	0.286	0.314	0.460	0.273	0.260	0.417	1.000
	P-value	0.622	0.006	0.010	0.012	0.007	0.000	0.017	0.026	0.000	.
	n	78	75	78	77	73	76	76	73	76	78

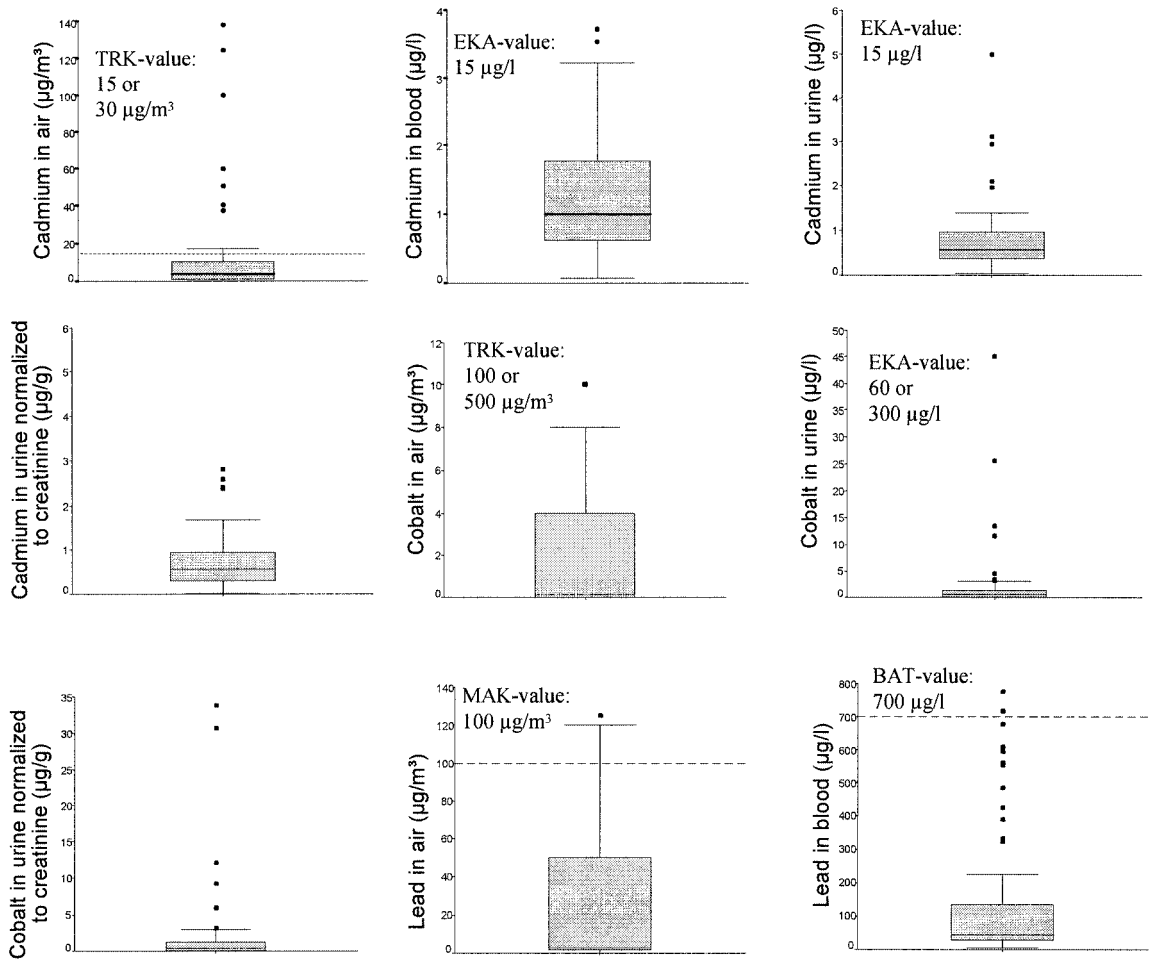

Fig. 1. Exposure scenario of the examined population: cadmium concentrations in air, blood and urine, cobalt concentrations in air and urine as well as lead concentrations in air and blood of 78 individuals were summarized. In addition the currently valid occupational-medical threshold limit values in Germany (TRK-, EKA-, MAK-, and BAK-values) are given with the exception of cobalt and cadmium in urine normalized to creatinine, since the German threshold value is defined without adjustment to creatinine. Threshold limit values of cadmium as well as cobalt in air depend on the type of exposure. Thus, two values are given. The horizontal line in the middle of a box shows the median of the sample. The edges of a box mark the 25th and 75th percentiles. The whiskers show the range of values that fall within 1.5 box-lengths. Values >1.5 box-lengths from the 25th or 75th percentiles are marked by a dot.

be influential in block 1. The first logistic regression model and both blocks of the second logistic regression model were built using a forward and backward stepwise selection using the score test with a *P*-value of 0.05 as inclusion criterion and the Wald test with a *P*-value <0.10 as exclusion criterion. Possible interactions between the relevant influential factors were evaluated by a logistic regression model with two blocks, the first block containing all parameters found to be influential in the models above and the second block containing all possible interactions between these influential parameters. Stepwise forward selection was used for the second block. Odds ratios were calculated as the ratios between the odds of exposed persons and the odds of individuals without exposure to cadmium, cobalt and lead. Model assessment was performed to evaluate the influence of single subjects on the estimation of the regression parameters. The δβs for each subject and each regression parameter were calculated for the final logistic regression model. All analyses are regarded as explorative. Therefore, no significance level is given.

Results

Exposure to cadmium, cobalt, lead and nickel

The concentrations of cadmium, cobalt and lead in the air of the working places of the 78 individuals examined in this study varied widely ranging from 0.05 to 138.00 µg/m^3 for cadmium, from 0 to 10 µg/m^3 for cobalt and from 0 to 125 µg/m^3 for lead (Figure 1). The median concentration of cadmium in the air of the working place was 3.8 µg/m^3 (1st and 3rd quartiles: 1.0 and 10.2 µg/m^3). Eleven of the 78 individuals were exposed to cadmium air concentrations higher than the TRK-value (the concentration that should not be exceeded due to German regulations) of 30 µg/m^3. Median cadmium concentrations in blood and urine were 1.0 (0.6; 1.8) µg/l and 0.6 (0.4; 1.0) µg/l, respectively. The median cobalt concentration in the air of the working places was 0.2 (0.0; 4.0) µg/m^3. Cobalt was not detectable in the air of the working places of 33 individuals. Even the highest cobalt exposure of 10 µg/m^3 was much smaller than the TRK-value of 100 or 500 µg/m^3 (the TRK-value of cobalt differs, depending on the type of production). Median lead concentrations in the air of the working places and in blood were 3.0 (1.6; 50.0) µg/m^3 and 44.1 (28.4; 136.8) µg/l, respectively. Lead air concentrations of three individuals and lead blood concentrations of two individuals exceeded the MAK-value for lead in air (100 µg/m^3) and the BAT-value (the concentration that should not be exceeded due to German regulations) for lead in blood (700 µg/l), respectively. Nickel was detectable in the air of the working places of only four individuals, ranging from 5 to 24 µg/m^3. Thus, nickel seems to be of minor relevance for the examined population.

Non-parametric Spearman's rank-correlation analysis

Correlation of DNA-SSB with exposure to heavy metals

Cadmium in air (R = 0.371; $P = 0.001$) and in blood (R = 0.220; $P = 0.053$) correlated with the extent of DNA-SSB in mononuclear blood cells of exposed subjects (Table I). Surprisingly, an even better correlation was found between cobalt air concentrations and the level of DNA-SSB (R = 0.401; $P < 0.001$). Similarly, cobalt in urine (normalized to creatinine) correlated with DNA-SSB (R = 0.381; $P = 0.001$). No correlation of DNA-SSB with lead in air or lead in blood could be observed (Table I).

Correlations between different types of exposure

As expected cobalt in air correlated with cobalt in urine (normalized to creatinine, R = 0.504; $P < 0.001$; Table I). Similarly, lead in air correlated with lead in blood (R = 0.417; $P < 0.001$). Cadmium in blood correlated with cadmium in urine (R = 0.499; $P < 0.001$). However, no correlation was observed between cadmium in air and cadmium in blood or urine (Table I). The lack of correlation between cadmium in air and blood might be explained by the smoking status, since cigarette smoking (smokers versus non-smokers) increased cadmium concentrations in blood and urine (Mann–Whitney U-tests for unpaired data: $P < 0.001$ and $P = 0.001$, respectively). Thus, cigarette smoking was a stronger determinant of cadmium blood and urine concentrations than cadmium air concentrations at the working place. In addition, cadmium concentrations in urine increased with age ($P = 0.001$, R = 0.361, Spearman-test), whereas only a borderline significant correlation was observed between cadmium concentrations in blood and age ($P = 0.061$, R = 0.215, Spearman-test). Several correlations between exposure to the examined heavy metals could be observed (Table I). For instance, cadmium in air correlated with cobalt and lead in air; cobalt in air correlated with lead in air and lead in blood. Since both, cadmium and cobalt correlate with the extent of DNA-SSB and additionally cadmium and cobalt correlate with each other (Table I), it is difficult to differentiate, whether both or which of the two heavy metals is responsible for the observed DNA damage. Therefore a logistic regression analysis was performed.

Logistic regression analysis

Determination of the cutpoint for DNA strand breaks

To define a range of 'normal' and 'increased' levels of DNA strand breaks a cutpoint had to be determined. For this purpose mean and standard deviation of DNA strand breaks of the control group were used to calculate a one-sided 90% confidence interval for non-exposed persons. This strategy seems to be acceptable since the distribution of DNA single strand breaks of the control group was symmetrical (skewness: 0.203) and therefore the assumption of normal distribution is plausible. Out of this a cutpoint of 1.17 (normalized elution rate that represents relative units for DNA-SSB) was obtained. This result closely resembles cutpoints from previous studies with much higher numbers of controls (22,37).

Determination of relevant parameters of influence (model finding)

Using multivariable logistic regression models the influence of the following parameters on DNA-SSB (dichotomized into 'normal' and 'increased') was examined: age, gender, cigarette pack years, alcohol consumption, iron in serum, cadmium in blood, cadmium in air, lead in air, lead in blood, cobalt in air and cobalt in urine. To determine the relevant parameters of influence a forward and a backward stepwise variable selection was used. Both selection strategies resulted in the same model containing cobalt in air ($P = 0.005$), cadmium in air ($P = 0.027$), cadmium in blood ($P = 0.016$) and lead in blood ($P = 0.049$). The corresponding *P*-values and 95% confidence intervals for the latter parameters are summarized in Table II. All other parameters were not accepted by the model. The univariable evaluation of all regarded parameters of influence by the score test at the beginning of model building can be found in Table III. *P*-values in the univariable evaluation smaller than 0.05 were obtained for cobalt in air ($P < 0.001$), cadmium in air ($P = 0.002$) and cadmium in blood ($P = 0.047$).

Since the influence of cobalt in air was surprising, we applied specific statistical models to find out whether cobalt in air gives additional information after preferentially considering all other possible parameters of influence. For this purpose a logistic regression model with two blocks was performed. The first block contained all mentioned influential parameters besides cobalt. The second block contained cobalt adjusted for all the parameters found to be influential in block 1. Both blocks were examined by forward and backward stepwise variable selection using the score test with a *P*-value of 0.05 as inclusion criterion and the Wald test with $P < 0.05$

Table II. Logistic regression model without interactions

A. Explanatory factors accepted by the logistic regression model:

Parameter	Odds ratio (OR)	95% Confidence interval of OR	*P*-value of the Wald test
Cobalt in air	1.472	1.126–1.924	0.005
Cadmium in air	1.041	1.004–1.078	0.027
Cadmium in blood	3.828	1.282–11.429	0.016
Lead in air	0.964	0.930–1.000	0.049

B. All other parameters analyzed were not regarded as explanatory by the model:

Parameter	*P*-value of the Score test
Age	0.818
Gender	0.674
Cigarette pack years	0.722
Alcohol consumption	0.768
Iron in serum	0.726
Lead in blood	0.433
Cobalt in urine	0.701

Table III. Multivariable evaluation of 11 possible parameters of influence on DNA-SSB by the score test at the beginning of the logistic regression procedure. Parameters resulting in *P*-values >0.05 are printed bold

Parameter	*P*-value of the score test
Age	0.448
Gender	0.697
Cigarette pack years	0.726
Alcohol consumption	0.719
Iron in serum	0.537
Cadmium in blood	**0.047**
Cadmium in air	**0.002**
Lead in air	0.140
Lead in blood	0.361
Cobalt in air	**<0.001**
Cobalt in urine	0.057

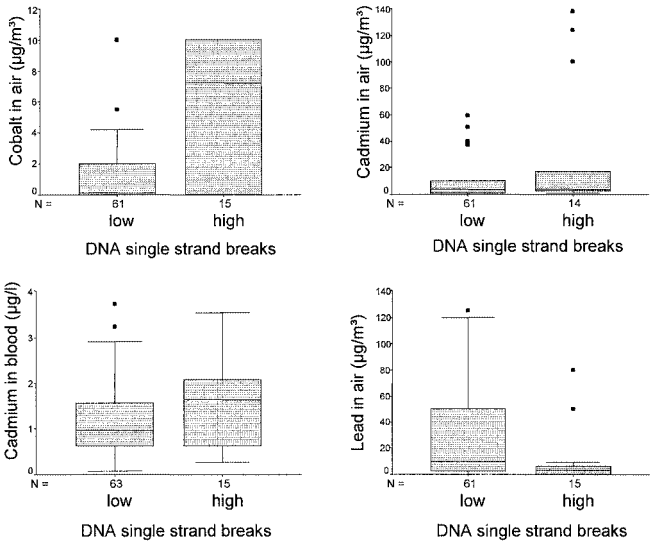

Fig. 2. Association of heavy metal exposures with DNA-SSB in mononuclear blood cells of 78 individuals. The horizontal line in the middle of a box shows the median of the sample. The edges of a box mark the 25th and 75th percentiles. The whiskers show the range of values that fall within 1.5 box-lengths. Values >1.5 box-lengths from the 25th or 75th percentiles are marked by a dot. Normalized elution rates are given as a measure for DNA-SSB.

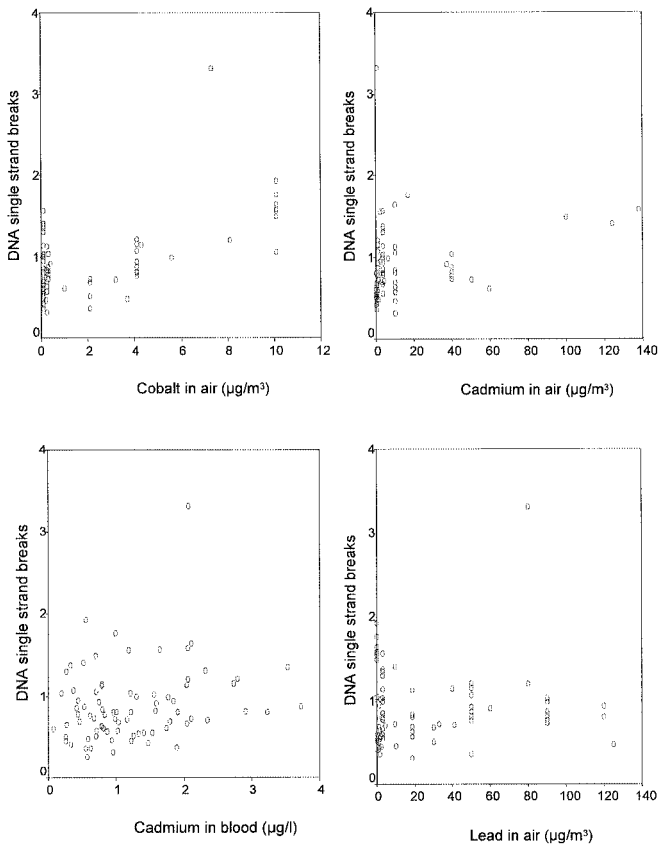

Fig. 3. Scatter plot analysis of cobalt in air, cadmium in air, cadmium in blood and lead in air versus DNA-SSB (expressed as normalized elution rates) in mononuclear blood cells of 78 individuals.

as exclusion criterion. Once again all four strategies resulted in the same model described above.

The influence of the four main parameters obtained by logistic regression analysis, namely cobalt in air, cadmium in blood and air as well as lead in air was visualized by box plots (Figure 2). Individuals with high levels of DNA-SSB were more often exposed to higher concentrations of cobalt in the air of the working place compared with individuals with low levels of DNA-SSB. A similar scenario was observed for cadmium in air and cadmium in blood. In contrast, individuals with high levels of DNA-SSB were more often exposed to lower levels of lead in air compared with individuals with lower levels of DNA damage. Scatter plots of the four main parameters are given in Figure 3 showing the influence of cobalt in air on the level of DNA-SSB. An increase in DNA-SSB can be seen, particularly for individuals exposed to >6 µg cobalt per m^3.

In order to identify possible artifacts that might cause a false positive effect in the group of highly exposed individuals with cobalt exposures >6 µg/m^3, we examined data from the controls (individuals without occupational exposure to heavy metals) that had been isolated and analyzed in parallel with the highly (>6 µg/m^3) exposed individuals. Mean values of individuals from the control group that have been isolated and analyzed in parallel with the highly exposed individuals were similar to the mean value of all other controls. Thus, an experimental artifact seems very improbable.

Evaluation of possible interactions between influential factors

To evaluate all possible interactions between the four relevant influential variables described above (Table III) a forward stepwise variable selection was used to add the relevant interactions to the logistic regression model. A final model with the main covariates and an interaction between cadmium in air and lead in air was obtained (Table IV). Using 0.5 as cutpoint for predicting an increased level of DNA-SSB 91% of the examined subjects were classified correctly.

Table IV. Logistic regression model with interactions

Parameter	Odds ratio (OR)	95% Confidence interval of OR	*P*-value of the Wald test
Cobalt in air	1.286	0.999–1.655	0.051
Cadmium in air	1.040	1.005–1.077	0.024
Cadmium in blood	3.111	1.057–9.154	0.039
Lead in air	0.861	0.749–0.990	0.035
Cobalt in air * lead in air	1.023	0.998–1.050	0.073

Parameters not a accepted by the Wald test:

Interactions	*P*-value of the Score test
Cadmium in blood * Cobalt in air	0.365
Cadmium in air * Cobalt in air	0.848
Cadmium in blood * Cadmium in air	0.467
Cadmium in blood * Lead in air	0.991
Cadmium in air * Lead in air	0.788
Cadmium in blood * Cadmium in air * Cobalt in air	0.961
Cadmium in blood * Cobalt in air * Lead in air	0.893
Cadmium in air * Cobalt in air * Lead in air	0.470
Cadmium in blood * Cadmium in air * Lead in air	0.574
Cadmium in blood * Cadmium in air * Cobalt in air * Lead in air	0.495

Interactions are indicated by * .

Risk evaluation by the logistic regression model: examples

Finally statistical analysis resulted in a model including cobalt in air, cadmium in air, cadmium in blood, lead in air and an

Table V. The influence of cobalt in air (μg/m^3), cadmium in air (μg/m^3), cadmium in blood (μg/l) and lead in air (μg/m^3) on the odds of having an increased level of DNA-SSB

Example no.	Cobalt in air (μg/m^3)	Cadmium in air (μg/m^3)	Cadmium in blood (μg/l)	Lead in air (μg/m^3)	Odds ratio
1	8.0	3.8	1.0	0.0	27.0
2	8.0	3.8	1.0	1.6	28.4
3	8.0	3.8	1.0	3.0	29.8
4	8.0	3.8	1.0	50.0	135.5
5	8.0	3.8	1.0	100.0	679.8
6	4.0	10.2	1.8	50.0	1.7
7	5.0	10.2	1.8	50.0	6.7
8	6.0	10.2	1.8	50.0	26.9
9	7.0	10.2	1.8	50.0	107.7
10	8.0	10.2	1.8	50.0	431.8

The odds ratio was calculated using the model: Odds ratio = $1.286^{\text{Co in air}} \times 1.040^{\text{Cd in air}} \times 3.111^{\text{Cd in blood}} \times 0.861^{\text{Pb in air}} \times 1.023^{\text{Co in air} \times \text{Pb in air}}$. The odds ratio gives the factor by which the odds for having an increased level of DNA-SSB is increased compared with a (constructed) individual without any exposure. To demonstrate the complex interactions between various types of exposure odds ratios for ten different exposure scenarios were calculated.

interaction of cobalt and lead in air. The odds ratios of interest here are the ratios of odds having increased levels of DNA-SSB between an exposed and an unexposed individual. The odds ratio in the model discussed in this paper is calculated as: $1.286^{\text{Co in air}} \times 1.040^{\text{Cd in air}} \times 3.111^{\text{Cd in blood}} \times 0.861^{\text{Pb in air}} \times 1.023^{\text{Co in air} \times \text{Pb in air}}$. For better understanding of this model the odds ratio for individuals with 'typical' exposures was calculated (Table V). Examples 1–5 demonstrate the influence of varying lead air concentrations (1.6–100 μg/m^3). A scenario with median values for cadmium in air (3.8 μg/m^3) and blood (10 μg/m^3) was chosen. Cobalt in air was set to a relatively high value with 8.0 μg/m^3. However, Figure 3 shows that 8.0 μg/m^3 is still within the level of exposure observed in the present study. Modeling these conditions demonstrates that lead air concentrations of 1.6 and 3.0 μg/m^3, the 25 and 50% percentiles in the present study, do not substantially increase odds ratios compared with the same scenario without lead exposure (Table V, examples 2 and 3). However, increasing lead air concentrations to the 75% percentile of 50 μg/m^3 or even to 100 μg/m^3 leads to a 4.7- or 23.9-fold increase in odds ratios compared with 1.6 μg/m^3 lead in air (Table V, examples 4 and 5). It should be considered that the strong influence of lead on DNA-SSB depends on the presence of cobalt, whereas lead alone does not influence levels of DNA-SSB. Another exposure scenario was chosen for examples 6–10 (Table V), where cadmium concentrations in air and blood as well as lead concentrations in air were set to the respective 75% percentiles obtained in the present study. Increasing cobalt air concentrations in the relatively narrow range between 4 and 8 μg/m^3 led to a strong increase in odds ratios from 1.67 to 431.8 (Figure 5, examples 6–10). It is important to consider that the model built under the condition of the present study cannot be exceeded beyond the limits of the observed levels of exposure. In addition the model is not suitable to assess exposures to single heavy metals, since it was constructed for a population of co-exposed individuals.

Model assessment

To evaluate the influence of single subjects on the estimation of the regression parameters the δβs for each subject and each regression parameter were calculated. No relevant change of the regression parameters by omitting a single subject could be observed for cobalt in air, lead in air and cadmium in blood. However, for cadmium in air omission of patient no. 10, a patient with high level DNA-SSB, no detectable cobalt in air, 124.1 μg/m^3 cadmium in air, 0.52 μg/l cadmium in blood and 9.4 μg/m^3 lead in air, would decrease the regression parameter for cadmium in air from 1.040 to 1.013. Thus, after omission of patient no. 10 an increase in cadmium by 1 μg/m^3 would no longer increase the odds by 4.0% but by only 1.3%. To assess the quality of prediction by the model cross validated probabilities were calculated for each subject, meaning that the probability of the respective subject for having increased levels of DNA-SSB is calculated after omitting the subject from model building having a positive prediction if and only if the calculated probability is >50%. With only one exception the false predictions were made for subjects with relatively low exposures to heavy metals that nevertheless had high levels of DNA-SSB.

Trypan blue exclusion

Analysis of trypan blue exclusion may be important for interpretation of alkaline elution data, since DNA-SSB may be caused by two basic mechanisms: (i) direct interaction of chemicals with DNA without substantially damaging the cell membrane and protein structures; this scenario results in increased elution rates and unaltered trypan blue exclusion; and (ii) damage to the cell membrane causing release of endonucleases from lysosomes that induce DNA-SSB as a secondary event due to cell membrane damage. The latter mechanism will result in increased DNA-SSB and diminished trypan blue exclusion. Of course both mechanisms may occur simultaneously. Thus, we determined trypan blue exclusion in all mononuclear blood cell preparations that were examined by alkaline elution. A correlation was obtained between trypan blue exclusion and normalized elution rates (P = 0.001, R = –0.511), indicating that an increase in DNA-SSB *in vivo* was associated with decreased trypan blue exclusion. However, only slight trends were obtained between trypan blue exclusion and cadmium air concentrations (P = 0.118, R = –0.182) as well as cobalt air concentrations (P = 0.066, R = –0.220). Thus, as expected DNA-SSB represent a more sensitive end point for biological effect monitoring.

Two-dimensional gel electrophoresis

Protein expression of individuals exposed to cobalt air concentrations of 10 μg/m^3 versus individuals without detectable cobalt exposure was compared by two-dimensional gel electrophoresis. A typical result is shown in Figure 4. Qualitative and apparent quantitative alterations in protein expression patterns in the highly exposed group were selective and certainly applied to <0.1% of all proteins.

Discussion

In this study we examined individuals co-exposed to several heavy metals, namely cadmium, cobalt and lead, since little is known about interactions between several heavy metals in humans. We observed that co-exposure to several heavy metals causes genotoxic effects that clearly exceed the sum of effects due to single heavy metals. However, before reporting about

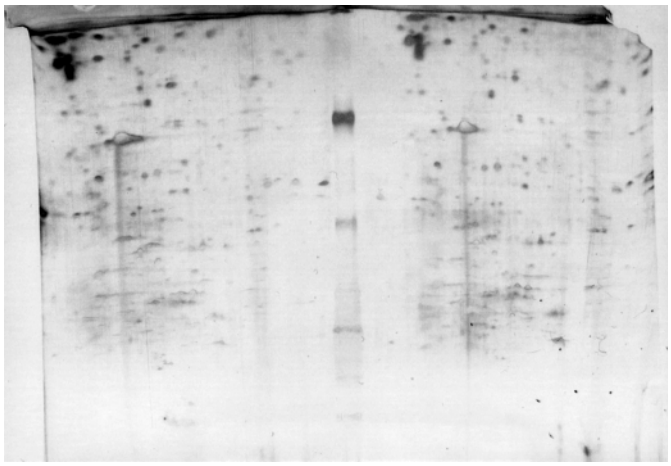

Fig. 4. Representative result from two-dimensional gel electrophoresis using protein extracts from mononuclear blood cells of an individual exposed to cobalt air concentrations of 10 μg/m^3 (left side) and an individual without detectable cobalt exposure (right side).

interactions, the influence of only single exposures to cadmium, cobalt or lead will be discussed.

Correlations with DNA-SSB were observed between cadmium concentrations in the air as well as cadmium concentrations in blood ($P = 0.001$ and $P = 0.053$, respectively), although the coefficients of correlation were relatively low ($R = 0.371$ and $R = 0.220$, respectively). Surprisingly a very good correlation was observed between cobalt air concentrations and DNA-SSB ($P < 0.001$; $R = 0.401$). Similarly, cobalt in urine correlated with DNA-SSB ($P = 0.001$; $R = 0.381$). This situation is surprising, since cobalt exposure in our study was relatively low (range: 0–10 μg/m^3 air) compared with the TRK-value (the concentration that should not be exceeded due to German regulations) of 100 μg/m^3. At a first glance this result seems to be in contrast to a recently published study that reported an absence of significant genotoxicity in mononuclear blood cells of workers ($n = 35$) exposed to cobalt containing dust (38). The alkaline comet assay and the micronucleus test were used in this study. A mean urinary concentration of 20 μg of cobalt per gram of creatinine was measured, which is equivalent to an airborne exposure level of 20 μg/m^3 (38). However, the important difference of our study is the co-exposure to other heavy metals: cobalt exposed individuals were co-exposed to cadmium and lead. Concentrations of lead in air and blood did not correlate with DNA-SSB in non-parametric correlation analysis. Only four of the 78 individuals examined in our study were exposed to detectable concentrations of nickel, indicating that nickel was of minor relevance for the studied population.

Interpretation of the observed correlations between cadmium as well as cobalt concentrations with DNA damage is difficult, since there are correlations between cobalt air concentration with cadmium concentrations in air and blood ($P = 0.021$, $R = 0.267$ and $P = 0.069$, $R = 0.210$, respectively). Thus, the increase in DNA-SSB may be explained by several mechanisms: (i) both cobalt and cadmium induce the observed DNA damage; (ii) only one of the two heavy metals causes the observed DNA damage, whereas exposure to the second metal represents an epiphenomenon; and (iii) DNA damage is induced by a third substance that correlates with cadmium and cobalt exposure, whereas cobalt and cadmium represent epiphenomena. The latter explanation seems to be extremely improbable, since the presence of another relevant genotoxic substance at the working places examined in this study can be excluded with high probability.

To be able to identify independent factors of influence on DNA damage and to analyze possible interactions we performed a logistic regression analysis. First we had to define a cutpoint differentiating between 'normal' and 'increased' levels of DNA-SSB. For this purpose we calculated a one-sided 90% confidence interval for the control subjects in our study. This strategy might be criticized, since the number of controls was relatively small. However, the resulting cutpoint of 1.17 (relative units) was plausible, since very similar data were obtained in previous studies with much higher numbers of individuals (37,22). Using this cutpoint in a multivariable logistic regression model only cobalt in air, cadmium in air, cadmium in blood and lead in blood proved to be of relevant influence on the level of DNA-SSB. We are aware of the fact that the positive result with cobalt is surprising, since cobalt exposure in our study (range: 0–10 μg/m^3) was smaller than the present TRK-value of 100 or 500 μg/m^3. To examine whether our positive result with cobalt is stable, we applied four different logistic regression strategies with two blocks, where all other parameters were considered preferentially compared with cobalt. However, all four strategies resulted in the same model described above.

To examine possible interactions between the latter four relevant influential parameters a logistic regression model was calculated that, besides the four single main variables, included all possible interactions between them. Indeed, an interaction between cadmium in air and lead in air was shown to be an additional factor of influence. Finally statistical analysis resulted in the following model that ideally summarizes the results: Odds ratio $= 1.286^{\text{Co in air}} \times 1.040^{\text{Cd in air}} \times 3.111^{\text{Cd in blood}} \times 0.861^{\text{Pb in air}} \times 1.023^{\text{Co in air} \times \text{Pb in air}}$, whereby odds ratio represents the ratio of odds for having an increased level of DNA-SSB between an exposed and unexposed individual. This model correctly predicts an increased level of DNA-SSB in 91% of the subjects of our study. For better understanding of this model typical exposure scenarios were modeled, showing that co-exposure to lead may have important effects. However, although lead alone did not increase levels of DNA-SSB co-exposure of cobalt with 8 μg/m^3 and lead with 100 μg/m^3 increased odds ratios by >20-fold compared with individuals exposed to cobalt alone. The influence of lead on cobalt induced DNA-SSB was concentration-dependent. According to the model, lead air concentrations of 50 μg/m^3 increased DNA-SSB ~5-fold, whereas no substantial influence of lead in air was calculated for concentrations of 3.0 and 1.6 μg/m^3. (It should be considered that the odds ratio is not the factor by which the number of DNA-SSB is increased, but gives the risk for having an increased level of single strand breaks compared with an unexposed person.) The formula given above for the odds ratio has to be considered with care since it cannot be exceeded beyond the levels of exposure observed in the current study. The model was constructed for individuals co-exposed to cadmium, cobalt and lead. Thus, conclusions for exposures to single heavy metals may be problematic. Theoretically, the model would predict a 'protective' effect of lead exposure in the absence of cobalt exposure, due to the factor of $0.861^{\text{Pb in air}}$. However, the latter conclusion is not acceptable, since all individuals in the model were exposed to lead and cobalt simultaneously. In the case of co-exposure the factors $1.023^{\text{Co in air} \times \text{Pb in air}}$ and $1.286^{\text{Co in air}}$

compensate for the factor $0.861^{Pb\ in\ air}$. Moreover the range of the confidence intervals for the odds ratios has to be considered as well.

Besides the influence of cobalt, the most interesting result of our study is the observation that co-exposures to more than one of the heavy metals cadmium, cobalt or lead may cause a more than multiplicative increase in risk compared with exposure to each substance alone. What is the molecular mechanism? The groups of Beyersmann and Hartwig reported that some heavy metals may not only be genotoxic, but also inhibit repair of DNA damage (15,17,29,39,40). Inhibition of DNA repair may occur at much lower concentrations than direct genotoxic effects. Cobalt, for instance, is known to be directly genotoxic by generation of reactive oxygen species, but also inhibits DNA repair enzymes, such as nucleotide excision repair or removal of UV-induced pyrimidine dimers, the latter occurring already at non-cytotoxic concentrations. The observations of Beyersmann and Hartwig stimulated us to measure the activity of OGG1, an enzyme critical for the susceptibility to reactive oxygen species. Repair of 8-oxoguanine was determined in the same samples that were analyzed for DNA-SSB (Janssen *et al.*, in preparation). We used an assay that determines cleavage of a template (40-mer) that carries 8-oxoguanine at base position 16. This assay has been chosen, since cobalt is known to generate oxidative stress and causes formation of 8-oxoguanine (40). Repair activity for 8-oxoguanine correlated inversely with levels of DNA-SSB ($P = 0.001$; R = -0.427; Pearson-test). Interestingly, a similar dose response (5–10 μg/m^3) was observed for inhibition of 8-oxoguanine repair as for induction of DNA-SSB: repair activity for 8-oxoguanine decreased at concentrations >4 μg cobalt/m^3. This supports the hypothesis that DNA damage in the exposed individuals might also be a secondary event due to DNA repair inhibition. DNA repair inhibition might also explain interactions between heavy metals, since a decreased repair capacity will increase susceptibility to direct genotoxic effects. A detailed description of the 8-oxoguanine repair data will be presented elsewhere (Janssen *et al.*, in preparation).

Alternative to this explanation, it might be postulated that cobalt causes broad and unspecific effects, including interactions with a wide range of proteins and interference with the transcription machinery, possibly due to competition with Mg^{2+} or Zn^{2+}. Thus, we compared protein expression of individuals exposed to cobalt air concentrations ~10 μg/m^3 versus individuals without detectable cobalt exposure by two-dimensional gel electrophoresis. Qualitative and apparent quantitative alterations in protein expression patterns in the highly exposed group were selective and affected certainly <0.1% of all proteins. In conclusion, two-dimensional gel electrophoresis shows that there are no broad alterations of protein expression in cobalt-exposed individuals.

Trypan blue exclusion was determined for all mononuclear blood cell preparations analyzed for DNA-SSB. In contrast to DNA-SSB, trypan blue exclusion did not correlate significantly with cobalt or cadmium exposure. However, normalized elution rates (a measure for DNA-SSB) inversely correlated with trypan blue exclusion, showing that cells with high levels of DNA-SSB are more likely to lose cell membrane integrity, or vice versa, cells with damaged membranes may release endonucleases that degrade DNA. This constellation differs from our previous studies with individuals exposed to 'classical' genotoxic substances, such as ethylene oxide or *N*-nitrosodiethanolamine, for which genotoxicity is known to occur at much lower concentrations than cytotoxicity (41–43). In the latter studies we observed no correlations between DNA-SSB and loss of cell membrane integrity. The constellation in the present study – damage to DNA and to the cell membrane –

Table VI. Induction of DNA-SSB by various occupational exposures

Exposure	Increase in DNA-SSB compared with the respective controls	References
Workers without detectable cobalt exposure vs. exposed workers (4–10 μg/m^3)	97%	(Present study)
Application of chemotherapy (before vs. 24 h after application of 1000 mg/m^2 cyclophosphamide)	155%	(22)
Sterilization plant workers with exposure to ethylene oxide (<0.1 vs. >0.1 mg/m^3)	93%	(43)
Metal workers with exposure to *N*-nitrosodiethanolamine (<500 v*s.* >500 ng/m^3)	69%	(42)
Painters (exposed workers on Fridays vs. Mondays)	60%	(37)
Car refinishing spray painters (exposed workers on Fridays vs. Mondays)	48%	(45)
Fire fighters exposed to o-nitroanisole after an accident in a chemical plant (19 days after exposure vs. 3 months after the accident)	22%	(46)
Taxi drivers (Fridays vs. Mondays with a free weekend)	22%	(37)
Petrol pump attendants (Fridays vs. Mondays)	18%	(47)
Roofers exposed to bitumen-products at 350°C (exposed workers on Fridays vs. Mondays)	48%	(48)
Road paving workers exposed to bitumen-products at 150°C (exposed workers on Fridays vs. Mondays)	ns	(48)

No significant increases in DNA-SSB:
- Nurses handling antineoplastic drugs with adequate safety precautions
- Workers exposed to TCDD even with TCDD concentrations >500 ng/ml
- Medical students exposed to formaldehyde concentrations >0.6 mg/m^3
- Car mechanics
- Carpenters

Table VII. Cobalt exposure (from Beyersmann and Hartwig, 1992)

Average intake from food:	20 μg/day
Concentrations reported in cobalt industries	0.1–0.2 mg/m^3
Exposure in the present study (air concentrations)	
Minimum–maximum	0.00–10 μg/m^3
Mean value ± standard error	1.97 ± 0.36 μg/m^3
TRK-value in air	
Cobalt powder	0.5 mg/m^3
Other uses	0.1 mg/m^3

might be explained by the action of reactive oxygen species that attack DNA as well as membranes. However, it should be emphasized that the extent of cell membrane damage was small, since trypan blue exclusion exceeded 70% for all preparations of mononuclear cells. DNA-SSB were a much more sensitive indicator of cobalt exposure than the trypan blue exclusion test, suggesting occurrence of DNA strand breaks at lower concentrations of cobalt, but loss of membrane integrity at not much higher concentrations.

Scatter plot analysis of the association between cobalt in air and DNA-SSB showed an increase in DNA-SSB when exposure to cobalt exceeded 4 μg/m^3 (Figure 3). The extent of DNA-SSB in individuals exposed to cobalt air concentrations >4 μg/m^3 was increased by 97% compared with individuals without detectable cobalt exposure. In previous studies we examined the influence of several genotoxic substances on DNA-SSB *in vivo* using the same methods as in the present study. An apparently higher extent in DNA-SSB than in the present study was induced by a single course of chemotherapy with cyclophosphamide (1000 mg/m^3) and carboplatin (350 mg/m^3 (Table VI)). However, exposure to cobalt air concentrations >4.2 μg/m^3 increased the extent of DNA-SSB more than all other occupational exposures to genotoxic substances that induced significant levels of DNA damage in our previous studies (Table VI). For instance exposure to ethylene oxide increased the level of DNA-SSB by 93% (individuals exposed to concentrations >0.1 versus <0.1 mg/m^3) and exposure to *N*-nitrosodiethanolamine (>500 versus <500 ng/m^3) by 69%. Thus, occupational co-exposure to cobalt in combination with other heavy metals seems to represent a serious, presently underestimated health hazard.

Our data show that the hazard due to occupational cobalt exposure presently may be underestimated by the IARC: cadmium is classified as IA, whereas cobalt is classified as IIB (44). However, cobalt is quite possibly even a stronger and in existing occupational exposures more dangerous carcinogen in humans, since (i) we observed induction of DNA damage *in vivo* after exposure to cobalt air concentrations of ~10 μg/m^3 that are considerably lower than the TRK value of 100 μg cobalt/m^3 and clearly lower than concentrations reported to occur in cobalt industries (Table VII); (ii) cadmium, although present at concentrations higher than the TRK-value (30 μg/m^3), was not more relevant for induction of DNA damage than cobalt; and (iii) the genotoxic mechanisms of cadmium and cobalt – generation of reactive oxygen species and DNA repair inhibition – are similar for both heavy metals and differences between them are rather quantitative than qualitative (40); and (iv) external cobalt exposure correlated with cobalt urine concentrations, a scenario that could not be observed for cadmium. Of course DNA-SSB in mononuclear blood cells are only an indicator of genotoxic exposure and mononuclear blood cells are not cells of origin for carcinomas. However, since the observed DNA-SSB are most probably generated by reactive oxygen species that also represent the main carcinogenic principle, and since the DNA-SSB after cobalt exposure occurred in the same concentration range in which repair of 8-oxoguanine declined – which should be similar also in target cells of carcinogenesis – DNA-SSB in mononuclear blood cells seem to represent a biologically relevant indicator. In conclusion, co-exposure to cadmium, cobalt and lead causes interactive effects. Co-exposure may cause genotoxic effects even if concentrations do not exceed TRK-values.

Acknowledgements

This study was supported by the BMBF and the DFG (Center Grant 519, project no. B5).

References

1. International Agency for Research on Cancer (IARC) (1993) Meeting of the IARC working group on beryllium, cadmium, mercury and exposures in the glass manufacturing industry. *Scand. J. Work Environ. Health*, **19**, 360–363.
2. Potts,C.L. (1965) Cadmium proteinurea. The health of battery workers exposed to cadmium oxide dust. *Ann. Occup. Hyg.*, **8**, 55–61.
3. Kipling,M.D. and Waterhouse,J.A.H. (1967) Cadmium and prostatic carcinoma. *Lancet*, **1**, 730–731.
4. Sorahan,T. and Waterhouse,J.A.H. (1985) Cancer of the prostate among nickel–cadmium battery workers. *Lancet*, **1**, 459.
5. Hayes,R.B. (1997) The carcinogenicity of metals in humans. *Cancer Causes Control*, **8**, 371–385.
6. Elinder,C.G., Kjellstrom,T., Hogstedt,C., Andersson,K. and Spang,G. (1985) Cancer mortality of cadmium workers. *Br. J. Ind. Med*., **42**, 651–655.
7. Kazantzis,G., Lam,T.H. and Sullivan,K.R. (1988) Mortality of cadmium-exposed workers. A five-year update. *Scand. J. Work Environ. Health*, **14**, 220–223.
8. Sorahan,T., Lister,A., Gilthorpe,M.S. and Harrington,J.M. (1995) Mortality of copper cadmium alloy workers with special reference to lung cancer and non-malignant diseases of the respiratory system, 1946–92. *Occup. Environ. Med.*, **52**, 804–812.
9. Stayner,L., Smith,R., Thun,M., Schnorr,T. and Lemen,R. (1992) A dose-response analysis and quantitative assessment of lung cancer risk and occupational cadmium. *Ann. Epidemiol*., **2**, 335–338.
10. Jarup,L., Bellander,T., Hogstedt,C. and Spang,G. (1998) Mortality and cancer incidence in Swedish battery workers exposed to cadmium and nickel. *Occup. Environ. Med*., **55**, 755–759.
11. Mandel,R. and Ryser,H.J. (1984) Mutagenicity of cadmium in Salmonella typhimurium and its synergism with two nitrosamines. *Mutat. Res*., **138**, 9–16.
12. Mandel,R. and Ryser,H.J. (1987) Mechanisms of synergism in the mutagenicity of cadmium and N-methyl-N′-nitrosourea in Salmonella typhimurium: the effect of pH. *Mutat. Res*., **176**, 1–10.
13. Takahashi,K., Imaeda,T. and Kawazoe,Y. (1988) Effect of metal ions on the adaptive response induced by N-methyl-N-nitrosourea in *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, **157**, 1124–1130.
14. Takahashi,K., Suzuki,M., Sekiguchi,M. and Kawazoe,Y. (1992) Effect of metal ions on transcription of the ada gene which encodes O6-methylguanine-DNA methyltransferase of *Escherichia coli*. *Chem. Pharm. Bull. Tokyo*, **40**, 2483–2486.
15. Hartwig,A. and Beyersmann,D. (1989) Enhancement of UV-induced mutagenesis and sister-chromatid exchanges by nickel ions in V79 cells: evidence for inhibition of DNA repair. *Mutat. Res.*, **217**, 65–73.
16. Snyder,R.D., Davis,G.F. and Lachmann,P.J. (1989) Inhibition by metals of X-ray and ultraviolet-induced DNA repair in human cells. *Biol. Trace Elem. Res*., **21**, 389–398.
17. Hartwig,A. (1995) Current aspects in metal genotoxicity. *Biometals*, **8**, 3–11.
18. Hurna,E., Siklenka,P. and Hurna,S. (1997) Effect of selenium on cadmium genotoxicity investigated by micronucleus assay. *Vet. Med. Praha*, **42**, 339–342.
19. Dally,H. and Hartwig,A. (1997) Induction and repair inhibition of oxidative DNA damage by nickel(II) and cadmium(II) in mammalian cells. *Carcinogenesis*, **18**, 1021–1026.
20. Saplakoglu,U. and Iscan,M. (1997) DNA single-strand breakage in rat lung, liver and kidney after single and combined treatments of nickel and cadmium. *Mutat. Res*., **394**, 133–140.
21. Latinwo,L.M., Ikediobi,C.O., Singh,N.P., Sponholtz,G., Fasanya,C. and Riley,L.(1997) Comparative studies of in vivo genotoxic effects of cadmium chloride in rat brain, kidney and liver cells. *Cell. Mol. Biol*., **43**, 203–210.
22. Hengstler,J.G., Fuchs,J. and Oesch,F. (1992) DNA strand breaks and cross-links in peripheral mononuclear blood cells of human ovarian cancer patients during chemotherapy with cyclophosphamide/carboplatin. *Cancer Res*. **52**, 5622–5626.
23. Hengstler,J.G., Fuchs,J., Tanner,B., Oesch-Bartlomowicz,B., Hölz,C. and Oesch,F. (1997) Analysis of DNA single strand breaks in human venous blood: A technique which does not require isolation of white blood cells. *Environ. Mol. Mutagen.*, **29**, 58–62.
24. Lloyd,D.R., Carmichael,P.L. and Phillips,D.H. (1998) Comparison of the

formation of 8-hydroxy-2′-deoxyguanosine and single- and double-strand breaks in DNA mediated by Fenton reactions. *Chem. Res. Toxicol*., **11**, 420–427.
25. Snyder,R.D. (1988) Role of active oxygen species in metal-induced DNA strand breakage in human diploid fibroblasts. *Mutat. Res.*, **193**, 237–246.
26. O'Brien,P. and Salacinski,H.J. (1998) Evidence that the reactions of cadmium in the presence of metallothionein can produce hydroxyl radicals. *Arch. Toxicol*., **72**, 690–700.
27. Calsou,P., Frit,P., Bozzato,C. and Salles,B. (1996) Negative interference of metal (II) ions with nucleotide excision repair in human cell-free extracts. *Carcinogenesis*, **17**, 2779–27782.
28. Hartwig,A. (1998) Carcinogenicity of metal compounds: possible role of DNA repair inhibition. *Toxicol. Lett*., **28**, 235–239.
29. Hartmann,M. and Hartwig,A. (1998) Disturbance of DNA damage recognition after UV-irradiation by nickel(II) and cadmium(II) in mammalian cells. *Carcinogenesis*, **19**, 617–621.
30. Hirano,T., Yamaguchi,Y. and Kasai,H. (1997) Inhibition of 8-hydroxyguanine repair in testes after administration of cadmium chloride to GSH-depleted rats. *Toxicol. Appl. Pharmacol.*, **147**, 9–14.
31. Lynn,S., Lai,H.T., Kao,S.M., Lai,J. and Jan,K.Y. (1997) Cadmium inhibits DNA strand break rejoining in methyl methanesulfonate-treated CHO-K1 cells. *Toxicol. Appl. Pharmacol*., **144**, 171–176.
32. Stohs,S.J. and Bagchi,D. (1995) Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med*., **18**, 321–336.
33. Shimada,H., Shiao,Y.H., Shibata,M. and Waalkes,M.P. (1998) Cadmium suppresses apoptosis induced by chromium. *J. Toxicol. Environ. Health*, **54**, 159–168.
34. Hengstler,J.G., Utesch,D., Steinberg,P., *et al.*(2000) Cryopreserved primary hepatocytes as an *in vitro* model for the evaluation of drug metabolism and enzyme induction. *Drug Metab. Rev.*, **32**, 81–118.
35. Fritz,G., Hengstler,J.G. and Kaina,B. (1997) High-dose selection with mafosfamide results in sensitivity to DNA cross-linking agents: characterization of hypersensitive cell lines. *Cancer Res*., **57**, 454–460.
36. Rodemann,H.P. and Bayreuther,K. (1986) Differential degradation of [^{35}S]methionine polypeptides in Duchenne muscular dystrophy skin fibroblasts in vitro. *Proc. Natl Acad. Sci. USA*, **83**, 2086–2090.
37. Oesch,F., Hengstler,J.G. and Fuchs,J. (1994) Cigarette smoking protects mononuclear blood cells of carcinogen exposed workers from additional work-exposure induced DNA single strand breaks. *Mutat. Res*., **321**, 175–185.
38. De Boeck,M., Lardau,S., Buchet,J.-P., Kirsch-Volders,M. and Lison,D. (2000) Absence of significant genotoxicity in lymphocytes and urine from workers exposed to moderate levels of cobalt-containing dust: A cross-sectional study. *Environ. Mol. Mutagen.*, **36**, 151–160.
39. Hartwig,A., Snyder,R.D., Schlepegrell,R. and Beyersmann,D. (1991) Modulation by Co(II) of UV-induced DNA repair, mutagenesis and sister-chromatid exchanges in mammalian cells. *Mutat. Res*., **248**, 177–185.
40. Beyersmann,D. and Hartwig,A. (1992) The genetic toxicology of cobalt. *Toxicol. Appl. Pharmaco*l., **115**, 137–145.
41. Fuchs,J., Wullenweber,U., Hengstler,J.G., Bienfait,H.G., Hiltl,G. and Oesch,F. (1994) Genotoxic risk for humans due to a working place exposure to ethylene oxide: remarkable individual differences in susceptibility. *Arch. Toxicol*., **68**, 343–348.
42. Fuchs,J., Burg,J., Hengstler,J.G., Bolm-Audorff,U. and Oesch,F. (1995) DNA damage in mononuclear blood cells of metal workers exposed to N-nitrosodiethanolamine in cutting fluids. *Mutat. Res*., **342**, 95–102.
43. Fuchs,J., Hengstler,J.G., Jung,D., Hiltl,G., Konietzko,J. and Oesch,F. (1995) DNA damage in nurses handling antineoplasitic agents. *Mutat. Res*., **342**, 17–23.
44. International Agency for Research on Cancer (IARC) (1991) Chlorinated drinking-water; chlorination by-products; some other halogenated compounds; cobalt and cobalt compounds. International Agency for Research on Cancer Working Group, Lyon, 12–19 June 1990. *IARC Monogr. Eval. Carcinog. Risks Hum*., **52**, 1–544.
45. Fuchs,J., Hengstler,J.G., Boettler,G. and Oesch,F. (1996) Primary DNA damage in peripheral mononuclear blood cells of workers exposed to bitumen-based products. *Int. Arch. Occup. Environ. Health*, **68**, 141–146.
46. Hengstler,J.G., Fuchs,J., Bolm-Audorff,U., Meyer,S. and Oesch,F. (1995) DNA single strand breaks in fire fighters accidentally exposed to o-nitroanisole and other chemicals. *Scand. J. Work Environ. Health*, **21**, 36–42.
47. Oesch,F., Fuchs,J., Arand,M., Vaupel,J. and Hengstler,J.G. (1995) DNA single strand break analysis in mononuclear blood cells of gasoline station attendants. *Int. Arch. Occup. Environ. Health*, **67**, 35–39.
48. Fuchs,J., Hengstler,J.G., Hummrich,F. and Oesch,F. (1996) Transient increase in DNA strand breaks in car refinishing spray painters. *Scand. J. Work Environ. Health*, **22**, 438–443.

Received June 19, 2000; revised and accepted May 24, 2002