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Original article:

Immunotoxicity of co-exposures to heavy metals: *In vitro* studies and results from occupational exposure to cadmium, cobalt and lead

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ABSTRACT

Introduction: The influence of cobalt (Co), cadmium (cd) and lead (Pb) on the immune system of occupationally exposed persons was assessed. *Methods*: 36 persons exposed to Co, Cd and Pb were analyzed. Concentrations of the heavy metals in the air of the working places as well as concentrations in blood and urine of the exposed individuals were measured. Lymphocyte proliferation and *in vitro*-chromate resistance were determined. *Results:* In univariate analysis lymphocyte proliferation was correlated to Co in air (Spearman's R=-0.69, p<0.001), Co in urine (R=-0.48, p = 0.003), Pb in blood (R=-0.32, p = 0.058), Cd in air (R=-0.53, p = 0.001), and Cd in urine (R=-0.40, p=0.021). *In vitro*-chromate resistance of lymphocytes was correlated to Co in air (Spearman's R=-0.50, p=0.002), Pb in air (R=-0.29, p=0.099), Pb in blood (R=-0.42, p=0.012), Cd in air (R=-0.32, p=0.069), Cd in blood (R=-0.32, p=0.057), and Cd in urine (R=-0.37, p=0.035). The following potentially influential parameters were included into a multiple logistic regression analysis: Cd in air, in blood and in urine, Co in air and in urine, Pb in air and in blood, Fe in serum, smoking habits, alcohol intake, and age. Only two of them, namely Co in air and Pb in air, were shown to have an explanatory influence. *Discussion:* Exposures to Co and Pb in concentrations below or close to the current TLV lead to clear effects on lymphocyte proliferation and chromate resistance in exposed individuals. A synergistic effect could be shown. Besides the conventional lymphocyte proliferation assay the newly established chromate resistance assay improves identification of humans exposed to immunotoxic substances.

Keywords: immunology; lymphocyte; proliferation; chromate resistance; cadmium; cobalt; lead; metal; human; occupational exposure

Abbreviations: BEI: Biological Exposure Index, BrdU: bromodeoxyuridine, Cd: cadmium, Co: cobalt, Cr(VI): chromate(VI), ELISA: enzyme linked immunosorbent assay, FCS: fetal calf serum, MNC: mononuclear cells, Ni: nickel, Pb: lead, PHA: phytohemagglutinine, TLV: Threshold Limit Value, Zn: zinc

INTRODUCTION

Presently, only little is known about the influence of heavy metals (Co, Cr(VI), Cd, Pb or Ni) on the immune system in human populations (Hengstler et al., 2003). No studies on diseases possibly related to suppressive immunotoxicity except of cancer studies have been carried out. Evidence has been published that the humoral immune system might be susceptible to the exposure of Pb or Cd (Ewers et al., 1982; Hambach et al., 1983; Jaremin et al., 1990; Yucesoy et al., 1997a,b; Karakaya et al., 1994). In animal experiments as well as *in vitro* investigations Cd, Co or Cr(VI) suppress the ability of B- and T-cells to proliferate. The influence of Pb has been reported to be much weaker. In contrast, some investigators observed an enhancement in proliferation after exposure to Pb (Borella et al., 1990; Wang et al., 1996). The mechanisms causing these disturbances in proliferation may be due to DNA damage (Dally et al., 1997; Beyersmann et al., 1992; Hengstler et al., 2003) by production of oxygen radicals, but also to DNA repair inhibition (Dally et al., 1997; Hartwig et al, 1996; Kasten et al., 1997; Hartmann et al., 1997; Hengstler et al., 2003). However, different mechanisms seem to be responsible for the generation of reactive oxygen species, namely redox cycling for Cr(VI) and depletion of sulfhydryl groups for Cd (Stohs et al., 2000). The effect on DNA-repair of Co and Ni are competed by magnesium, whilst that of Cd is reversed by the addition of zinc (Otsuka et al., 1991; Hartwig et al., 1998). The activation of protein kinase by Cd and Cr(VI) and the induction of c-fos and c-jun by Cd, on the other hand, point to a stimulating effect of these metals on cell proliferation (Beyersmann et al., 1994; Bagchi et al., 1997; Beyersmann et al., 1997). The effect of Cd on cell proliferation has been reported to depend on dose, whereby lower concentrations (1 µM) lead to a stimulation and higher concentrations to a suppression of proliferation (McCabe et al., 1990). Having such a complex situation already for single metals, it seems impossible to predict the effect of a mixture of metals on humans from the experimental data. Synergistic effects on the immune system can be expected. In laboratory studies the extent of such interactions can be estimated (Nesnow et al., 1998). In field studies such estimations are not promising, if the influential substances do not share a common mechanism of action (Mumtaz et al., 1998; Van den Berg et al., 1998; Cassee et al., 1998; von Mach et al., 2002; Hengstler et al., 2002;2003; Arand et al., 2003).

In the present study, we investigated a group of metal workers occupationally exposed to a variety of metals including Pb, Cd and Co. As recommended by several experts (Mauderly et al., 1993; El-Masri et al., 1997; Eide et al., 1998) we used an integrated approach to achieve an overall measure of the immunotoxic load of the exposed persons.

METHODS

Study design

Thirty-six workers (Thirty-two males and four females) occupationally exposed to different metals participated in the study. They were employed in six different companies, which are involved in dye production, galvanisation and recycling of batteries and electric devices. The employees were mainly exposed to different extents of Cd, Pb and Co. Blood sampling, exposure analysis (work place analysis, measurement of Cd, Pb and Co in air, Cd and Pb in blood and Cd in urine) as well as determination of creatinine in urine and Fe in serum were performed by the laboratory of U. B.-A. and H.-G.B., the analysis of Co in urine in the laboratory of O. M.-P., and the analysis of proliferation and *in vitro*-chromate resistance in the laboratory of D. J.. Data were not linked together before finishing the analyses in the involved laboratories.

Metal analysis

Air Monitoring

Personal air monitoring was done by pulling 3.5 litres/min of air through membrane filters using special pumps (Gilian Hiflowsampler®, Wayne, NJ, USA). The contents of heavy metals in the dust collected were analyzed by electrothermal atomic absorption spectrometry. Detection limit for Cd in air is 0.05 µg/m³, for Pb in air 1.5 µg/m³ and for Co in air 0.7 µg/m³.

Biological Monitoring

All heavy metal analyses were done by electrothermal atomic absorption according to standard-addition procedure (DFG, 2000). Cd was analyzed in whole blood after deproteinization with nitric acid (detection limit 0.08 µg/m³). For detection of Cd diluted urine was acidified with nitric acid (detection limit 0.04 µg/l). Determining Pb venous blood was acidified and diluted with 0.1% (v/v) Triton X-100 solution. (detection limit 12 µg/l). For detection of Co urine was diluted with 0.1% (v/v) Triton X-100 (detection limit 0.1µg/l). Transferrin-bound iron was removed by guanidine and detergents in weakly acid solution and reduced to Fe^{2+} with ascorbic acid. The Fe^{2+} forms a red-coloured complex with ferrozine, which is then determined photometrically (detection limit 20µg/l). Creatinine in urine was determined in diluted urine by use of kinetic photometry measurement according to Jaffè's reaction (detection limit 2µg/l).

Immunological studies:

Proliferation assay

Isolation of mononuclear cells from peripheral blood was performed by density centrifugation on a Ficoll gradient (specific density 1.077 g/ml) (Biochrom Seromed, Berlin, Germany) with consecutive washing with phosphate-buffered saline (PBS) supplemented with 2% of bovine serum albumine (Sigma, St.Louis, USA) (Hengstler et al., 1992). Proliferation assays (Hengstler et al., 1999) were performed with 500,000 cells/ml in culture medium (RPMI 1640, 10% of fetal calf serum (FCS) or autologous serum, 4mMol glutamine, 50µg/ml streptomycin, 50U/ml penicillin) in microtiter plates. Proliferation was induced by phytohemagglutinin (PHA, (Biochrom Seromed®, Berlin) 2.4 µg/ml. For the assessment of lymphocyte proliferation a commercially available ELISA assay (Cell Proliferation ELISA (colorimetric)®, Boehringer Mannheim) was used. This assay measures bromodeoxyuridine (BrdU) incorporated into the DNA instead of thymidine during cell proliferation. BrdU is detected by anti-BrdU-FAB-fragments linked to peroxidase (POD). Tetramethylbenzidine.serves as substrate of POD.

In vitro studies:

In vitro studies were performed using freshly isolated mononuclear cells. The culture medium was supplied with FCS. To evaluate its immunotoxicity, ammonium dichromate(VI) was added to lymphocyte cultures in different concentrations (0, 70, 102, 150, 183, 221, 268, 325, 394, 477, 578 and 700 ng/ml). Alike Cd nitrate was added in concentrations of 0, 1, 1.58, 2, 2.58, 3.18, 4, 5.03, 6.33, 7.95 and 10 µg/ml. Cultures were replicated three times. Additionally, lymphocytes were co-exposed to all combinations of different ammonium dichromate(VI) (0, 183, 221, 325, 394, 477, 578 and 700 ng/ml) and Cd nitrate (0, 2, 3.18, 4, 5.03, 6.33 and 10 µg/ml) concentrations to measure the joint effect of both metals.

Ex vivo studies:

Workers' lymphocytes were processed by the same technique as described for the *in vitro* experiments except that cells and autologous serum were stored before use. After controlled freezing (10 million cells/ml in RPMI 1640, 10% fetal calf serum, 4 mMol glutamine, 50µg/ml streptomycin, 50 U/ml penicillin (all reagents from Biochrom Seromed, Berlin, Germany) supplemented with 7.5% dimethylsulfoxide (DMSO) (Sigma, St.Louis, USA) cells were stored in

liquid nitrogen (von Mach et al., 2003). Serum was stored at –80°C. After thawing cells were washed three times and taken into culture medium as described above. Autologous serum was used as supplement instead of FCS.

***In vitro-chromate resistance-test**:*
Proliferation studies were performed in culture media containing increasing concentrations of diammonium chromate (from 0 to 700 ppb). We assessed the concentration of chromate, that reduced proliferation capacity of the lymphocytes to one half of its maximum ($Cr_{p1/2}$) (Jung et al., 1998). The idea behind this "*in vitro*-chromate resistance-test" is to yield a surrogate for the extent of exposure of the individual to the sum of immunotoxic influences acting on the lymphocytes *in vivo*. The higher the Cr(VI) concentration was, the lymphocytes were able to withstand *in vitro*, the lower the preceding immunotoxic load *in vivo* was assumed.

Statistics

Univariate analysis
Standard descriptive statistics were calculated for describing the study population, the influential factors, the immunological parameters and for correlating them (minimum, maximum, quartiles, Spearman's rank correlation coefficient, Mann-Whitney test, χ^2-test). Covariates were subdivided into two or three categories, which were analyzed on as a categorical independent variable (**Table 1**). No information about clinically relevant cutpoints for the outcome variables "lymphocyte proliferation" and "*in vitro*-chromate resistance" are known. Therefore, they were dichotomized according to the median (optical density of 2.4 for lymphocoyte proliferation and 115 ng Cr(VI) / ml for the *in vitro*-chromate resistance). Due to this, the analyses have to be regarded as explorative. Associations were assessed by the Fisher´s exact test for 2x2 contingency tables and the χ^2-test for larger tables.

Multifactorial analysis
Logistic regression was applied to analyse the immunologic parameters' dependency on the covariates. Due to the limited number of data only three covariates were introduced in logistic regression at a time: (1.) Cd in air, in blood and in urine (adjusted to creatinine in urine) were tested together, (2.) Co in air and in urine and smoking, (3.) Pb in air and in blood and age, and (4.) alcohol and Fe in serum. Those covariates that proved to be of significant influence, were included into a final analysis. The forward stepwise selection with the likelihood-ratio criterion was used while performing the logistic regression. The inclusion criterion was $p \leq 0.05$ and the exclusion criterion was $p > 0.10$. Statistical analysis was performed with SPSS-package 8.0®.

RESULTS

***In vitro* effects of Cd and Cr(VI) on lymphocyte proliferation**
Human lymphocytes were incubated *in vitro* with Cd in concentrations ranging from 0 to 10 µg/ml and Cr(VI) in concentrations from 0 to 700 ng/ml. Both heavy metals inhibited lymphocyte proliferation in a dose-dependent manner (**Fig. 1a and b**). EC50 for Cd was 5.4 µg/ml. For Cr(VI) an EC50 of 390 ng/ml was obtained. However, incubation with 394 ng/ml of Cr(VI) and with 5.03 µg/ml of Cd (both concentrations close to the respective EC50) together resulted in a reduction of lymphocyte proliferation to 13% (**Fig. 2**). Addition of Cd systematically influenced the dose-dependent inhibition of proliferation by Cr (**Fig.2**). This observation led us to the hypothesis that the sensitivity of detection of immunotoxic effects can be improved by testing the *in vitro*-chromate resistance of lymphocytes that have been exposed to toxic substances *in vivo*. To test this hypothesis lymphocytes obtained from individuals exposed to various heavy metals were tested by a conventional proliferation assay as well as by the *in vitro*-chromate resistance test.

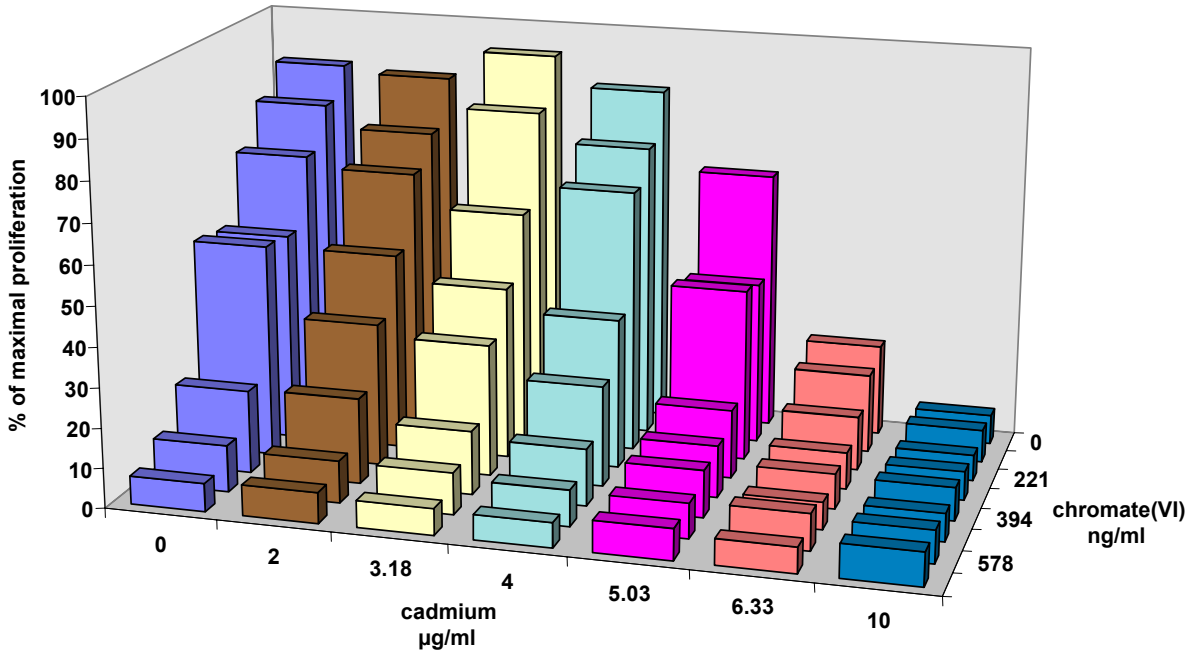

Fig. 2: Lymphocyte proliferation and co-exposure to chromate(VI) and cadmium: Lymphocytes were exposed to all combinations of different chromate(VI) and cadmium concentrations *in vitro*. Proliferation is depicted as % of maximal proliferation in the respective test.

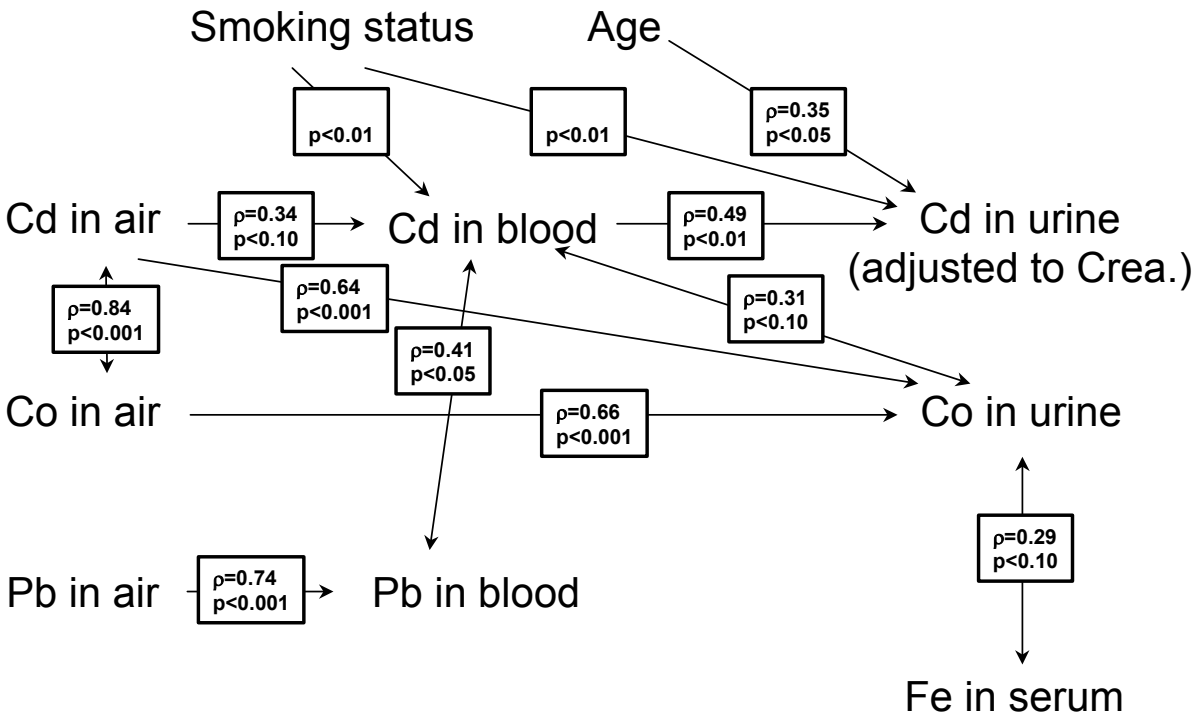

Fig. 3: Survey of the interdependences of influential parameters. The interdependences with the smoking status are given by the p-values of the Mann-Whitney test. All other interdependences are represented by Spearman's rank correlation coefficients and the affiliated p-values.

Exposure to heavy metals *in vivo*

Exposure to Co of the 36 persons ranged from 0 to 10 µg/m³ in air and from 0.1 to 45.1µg/l in urine. The concentrations of Co in air never exceeded the current TLV (Threshold Limit Value), in one person urine concentration was higher than the current BEI (Biological Exposure Index) (**Table 2**). Pb concentrations ranged from 0 to 125 µg/m³ in air and from 13.2 to 775.4 µg/l in blood. For ten persons the Pb concentrations in air reached or exceeded the currently accepted TLV of 50µg/m³, eleven had blood concentrations higher than the current BEI of 300µg/l (**Table 2**). Cd concentrations ranged from 0.1 to 138 µg/m³ in air, from 0.08 to 3.72µg/l in blood, and from 0.1 to 45.1µg/g creatinine in urine. Whilst all Cd concentrations measured in blood and urine where beyond the respective BEI's, the TLV in air was exceeded at the workplace of five persons.

Interdependences of Co, Pb and Cd

It should be considered that due to the workplace characteristics exposure to each heavy metal examined in this study was not independent from the exposure to the others. So Co exposure highly correlated with Cd in air ($r = 0.84$, $p<0.001$), but not with Pb in air ($r = -0.094$, $p = 0.597$). Pb and Cd in air were not correlated ($r = 0.051$, $p = 0.778$). In addition, concentration of Pb in blood was correlated with Cd in blood ($r=0.406$, $p = 0.014$). The interdependences of covariates are shown in figure 3. Pb in blood was dependent on Pb in air, as well as Co in urine on Co in air. Cd in air was only marginally associated with Cd in blood and not with Cd in urine. Cd in blood and urine were revealed to be interdependent. Cd in urine was associated with age. Moreover smokers had relevantly higher Cd concentrations in blood ($p = 0.006$) and in urine ($p = 0.038$) than non-smokers.

Univariate correlation analysis of exposure to heavy metals and lymphocyte function

In univariate analysis lymphocyte proliferation was correlated to Co in air (Spearman's $R = -0.69$, $p<0.001$), Co in urine ($R = -0.48$, $p = 0.003$), Pb in blood ($R = -0.32$, $p = 0.058$), Cd in air ($R = -0.53$, $p = 0.001$), and Cd in urine ($R = -0.40$, $p = 0.021$). *In vitro*-chromate resistance of lymphocytes was correlated to Co in air (Spearman's $R = -0.50$, $p = 0.002$), Pb in air ($R = -0.29$, $p = 0.099$), Pb in blood ($R = -0.42$, $p = 0.012$), Cd in air ($R = -0.32$, $p = 0.069$), Cd in blood ($R = -0.32$, $p = 0.057$), and Cd in urine ($R = -0.37$, $p = 0.035$). Neither *in vitro*-chromate resistance nor proliferation rate were associated with sex, age, current smoking status, or nationality ($p>0,10$ in the Mann-Whitney test). Spearman`s rank correlation coefficients are summarized in **Table 3**. Results of Fisher`s exact test respectively the χ^2-test using categorized variables are given in **Table 4**. The data show that all three heavy metals examined in the present study showed clear effects on lymphocyte proliferation and *in vitro*-chromate resistance. The highest correlation was obtained between Co in air and lymphocyte proliferation resulting in a coefficient of correlation of –0.69 ($p<0.001$). Since exposure to the three heavy metals was correlated to each other and all of them also correlated with lymphocyte proliferation and *in vitro*-chromate resistance, it is not possible to establish a cause-effect relationship based on univariate analysis. Thus, a multivariate analysis (logistic regression model) was applied.

		Fe (µg/dl)	Cd in urine adj. to creat. (µg/g)	Cd in blood (mg/l)	Cd in air (mg/m³)
N	valid	36	33	36	33
	missing	0	3	0	3
Minimum		49.0	0.1	0.08	0.02
Maximum		203.0	138.0	3.72	2.82
Percentile	25	84.0	0.4	0.59	0.31
	50	118.5	1.0	1.17	0.63
	75	140.8	3.6	1.88	1.21

		Pb in air (µg/m³)	Pb in blood (µg/l)	Co in air (µg/m³)	Co in urine (µg/l)
N	valid	34	36	34	36
	missing	2	0	2	0
Minimum		0.0	13.2	0.0	0.1
Maximum		125.0	775.4	10.0	45.1
Percentile	25	0.3	40.1	0.0	0.2
	50	2.2	83.3	3.8	0.8
	75	50.0	414.8	6.1	2.3

		Age (years)	Alcohol (g/day)	Max. proliferation	Cr resistance (ng/ml)
N	valid	36	36	36	36
	missing	0	0	0	0
Minimum		22	0.0	0.30	40
Maximum		58	76.0	5.16	427.5
Percentile	25	32	0.3	0.74	74.5
	50	37	3.5	2.37	116.0
	75	49	16.8	3.36	172.9

Tab. 2: Descriptive analysis of the data. The Threshold Limit Values (TLVs) in air and their corresponding Biological Exposure Indices (BEIs) in body fluids according to the National Institute of Occupational Health and Safety (NIOSH) and the American Conference of Governmental Industrial Hygienists (ACGIH) are 10 µg/m³ for Cadmium (Cd) in air, 50 µg/m³ for lead (Pb) in air and 20 µg/m³ for cobalt (Co) in air, 5 µg/l for Cd in blood, 300 µg/l for Pb in blood, 5 µg/g creatinine for Cd in urine and 15 µg/l for Co in urine.

		chromate resistance	age	pack years	alcohol	Fe in serum	Cd in air	Cd in blood	Cd in urine adj.	Pb in air	Pb in blood	Co in air	Co in urine
proliferation	r	**.569**	.031	.021	.194	**.369**	**-.531**	-.249	**-.401**	-.113	*-.319*	**-.694**	**-.478**
	p	**<.001**	.860	.901	.258	**.027**	**.001**	.143	**.021**	.525	*.058*	**<.001**	**.003**
chromate resistance	r		.021	-.001	.218	**.391**	*-.321*	*-.320*	**-.368**	*-.287*	**-.416**	**-.502**	-.168
	p		.901	.995	.201	**.018**	*.069*	*.057*	**.035**	*.099*	**.012**	**.002**	.327
age	r			**.407**	.217	-.159	-.029	-.022	**.354**	.187	.149	*-.318*	-.216
	p			**.014**	.205	.355	.872	.901	**.043**	.291	.386	*.066*	.206
packyears	r				*.304*	.061	-.062	**.409**	.260	.200	.204	-.226	-.087
	p				*.072*	.726	.730	**.013**	.144	257	.234	.198	.613
alcohol	r					.041	.059	.008	-.116	.189	.001	-.122	-.053
	p					.812	.744	.965	.522	.284	.996	.490	.761
Fe in serum	r						-.080	.000	-.275	-.069	-.245	-.160	*-.293*
	p						.660	.999	.121	.700	.151	.366	*.083*
Cd in air	r							*.335*	.183	.051	.180	**.840**	**.635**
	p							*.057*	.333	.778	.317	**<.001**	**<.001**
Cd in blood	r								**.494**	.278	**.406**	.279	*.311*
	p								**.003**	.112	**.014**	.111	*.065*
Cd in urine adj. to crea	r									.184	*.316*	-.007	-.013
	p									.323	*.073*	.969	.943
Pb in air	r										**.737**	-.094	.108
	p										**<.001**	.597	.543
Pb in blood	r											.134	.261
	p											.449	.125
Co in air	r												**.656**
	p												**<.001**

Tab. 3: Interdependence of different factors and their influence on lymphocyte proliferation and *in vitro*-chromate resistance. Data are Spearman's rank correlation coefficients (r) and the corresponding p-values.

		age	Smoking status	alcohol	Fe in serum
proliferation	p	.738	1	1	**.044**
chromate resistance	p	1	.738	.725	**.044**

		Cd in air	Cd in blood	Cd in urine adj. to creat.	Pb in air	Pb in blood	Co in air	Co in urine
proliferation	p	**.001**	1	**.038**	*.092*	.315	**<.001**	**.041**
chromate resistance	p	*.080*	.505	.169	**.013**	*.092*	**.032**	.500

Tab. 4: Influence of different factors on lymphocyte proliferation and *in vitro*-chromate resistance. Data are the p-values of Fisher`s exact test for 2x2 tables respectively the χ^2-test for larger tables

Multifactorial analyses

The following potentially influential parameters were included into multiple logistic regression analysis: Cd in air, in blood and in urine, Co in air and in urine, Pb in air and in blood, Fe in serum, smoking habits, alcohol intake, and age. Two of them, namely Co in air and Pb in air, were shown to have an explanatory influence on the immune parameters under investigation. Exposure to Co in air was a variable influencing lymphocyte proliferation (OR: 50; 95%-confidence interval: 5 – 515). Pb in air influenced *in vitro*-chromate resistance (OR: 7.6; 95%-confidence interval: 1.7 – 42.2). The large confidence intervals are due to the relatively small population being examined. In contrast, Cd did not come out to be an influential factor.

To illustrate the joined influence of Pb and Co on *in vitro* chromate resistance the exposed individuals were classified into three subgroups according to their metal exposure. In group 1 thirteen workers with low Pb exposure (<10µg/m³) and no detectable Co exposure were included, in group 2 seven workers with low Pb exposure, but measurable Co exposure (>0 µg/m³) and in group 3 fourteen workers with higher Pb exposure (>10µg/m³) and measurable Co exposure (>0 µg/m³) (**Fig 4**). A statistically significant decrease in chromate resistance was obtained from group 1 to group 2 (p=0.03). An even larger difference was observed between group 1 and group 3 (p=0.001). This result suggests that both Co and Pb contribute to depression of lymphocyte function.

Iron (Fe) in serum and its correlations to other covariates and immunological parameters

Fe in serum was positively correlated to lymphocyte proliferation (R = 0.37, p = 0.027) and *in vitro*-chromate resistance (R = 0.39, p = 0.018). Moreover, it was negatively correlated to Pb in blood (R = -0.25, p = 0.151), Co in urine (R = -0.29,

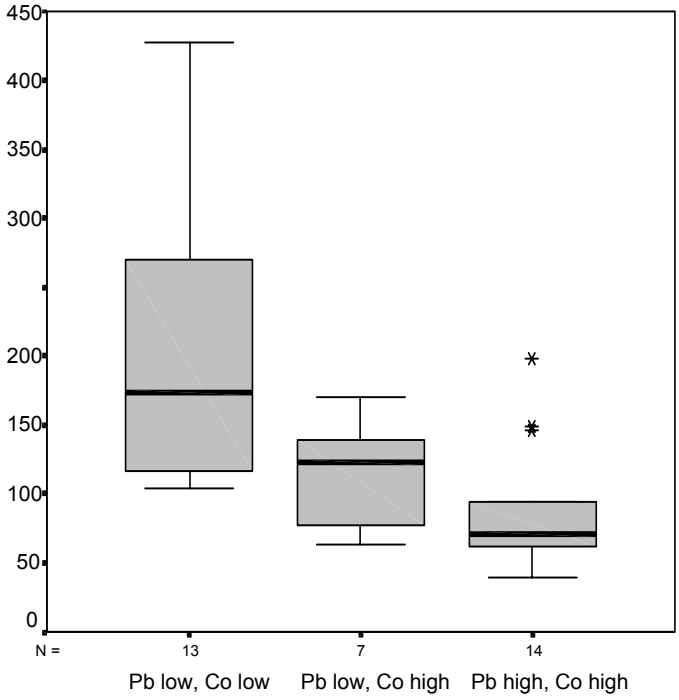

Fig. 4: Effect of cobalt (Co) and lead (Pb) in air and co-exposure to both heavy metals on *in vitro*-chromate resistance. Exposure to Co and Pb was dichotomized into low and high exposure using cutpoints of 10 µg/m³ for Pb and 0 µg/m³ for Co. The first subgroup includes thirteen, the second seven and the third fourteen persons. For the first subgroup median Pb exposure in air was 1.5 µg/m³, whereas median exposure to Co in air was 0 µg/m³. For the second group median Pb and Co exposures were 0 and 10 µg/m³, respectively. The third group was exposed to 50 µg/m³ Pb and 4 µg/m³ Co. The combination of high Pb and low Co exposure did not occur in our study population. The data show a clear influence of exposure to Co alone. Interestingly, co-exposure to both heavy metals, Co and Pb, led to an even stronger reduction of chromate resistance. Medians as well as lower and upper quartiles are shown. The Mann-Whitney test reveals a p-value of 0.03 between group 1 and 2, a p-value of 0.17 between group 2 and 3 and a p-value of 0.001 between group 1 and 3.

p = 0.083), and Cd in urine (R = -0.28, p = 0.121).

DISCUSSION

In this study we have shown that an *in vitro* chromate resistance test may allow a more sensitive identification of immunotoxic exposures than conventional lymphocyte proliferation assays. We observed that concentrations of both heavy metals, Cd or Cr(VI), that did not or only marginally reduce lymphocyte proliferation when given as single substances, caused a depression of lymphocyte proliferation well below 50%, when tested in combination. This led us to the hypothesis that addition of Cr(VI) to the *in vitro* system - in this study defined as *in vitro*-chromate resistance test - might be capable to detect an immunotoxic load *in vivo*, that could not be measured by the conventional lymphocyte proliferation assay. First promising experiences with this approach have been reported (Jung et al., 1998; 1997). A similar approach has been applied (Snyder et al., 1991) aiming to find sentinels for certain *in vivo* exposures by corresponding *in vitro* additives. Lymphocytes of rats exposed to Cd or Cr(VI) *in vivo* were more susceptible to exposure to the same than to the alternate metal *in vitro*.

We are aware of the fact that co-exposure *in vitro* to two heavy metals does not entirely reflect the successive mode of lymphocyte exposure in a biomonitoring strategy, where lymphocytes are first exposed to immunotoxic substances *in vivo* and later to Cr(VI) *in vitro*. In order to achieve a coexposure, incubation of lymphocytes from exposed individuals and exposure to Cr(VI) was performed by adding autologous serum, that still should contain the putative immunotoxic substances under study. To be able to compare sensitivity of the conventional lymphocyte proliferation assay and the newly established chromate resistance assay 36 individuals exposed to a combination of several heavy metals were examined by both assays.

A clear influence of Co and of Pb exposure was observed, if the combined information of both assays, lymphocyte proliferation and *in vitro*-chromate resistance, was taken into account. The influence of Pb in blood was relevant in the *in vitro*-chromate resistance assay but not in the conventional lymphocyte proliferation assay using univariate analysis. Also multivariate analysis including Cd in air, in blood and in urine, Co in air and in urine, Pb in air and in blood, smoking, age, alcohol and Fe in serum revealed a significant immunotoxic effect of Pb in air only in the *in vitro*-chromate resistance assay but not in the conventional lymphocyte proliferation assay. However, the opposite constellation was observed for Co exposure. The influence of Co in air on the *in vitro*-chromate resistance assay as well as on the conventional lymphocyte proliferation assay was shown in univariate analysis, whereas in multivariate analysis only Co in air proved to be an independent factor of influence in the conventional lymphocyte proliferation assay. The scenario clearly shows that the combined use of both assays, the *in vitro*-chromate resistance assay and the conventional lymphocyte proliferation assay, improves sensitivity of biomonitoring. The sole application of the lymphocyte proliferation assay would have underestimated the immunotoxic influence of Pb, whereas the sole use of the *in vitro*-chromate resistance assay wouldn't have identified the influence of Co in multivariate analysis. Moreover, it was possible to show an interaction between Co and Pb. However this interaction could be demonstrated only with the *in vitro*-chromate resistance assay but not with the conventional lymphocyte proliferation assay. The lowest degree of *in vitro*-chromate resistance was observed within the subpopulation with high Pb and high Co exposure. The subgroup with low exposure to both metals had the highest resistance against additional Cr(VI) *in vitro*. High exposure to only one of these two metals

(Co) resulted in an intermediate chromate resistance.

Originally, this study was focused to examine possible effects of occupational exposure to Cd. According to literature Cd could have been expected to be the most relevant immunotoxic heavy metal to which individuals of our study population were exposed (Hartwig et al., 1996; Beyersmann et al., 1997). Thus, it was surprising that our results showed that not occupational exposure to Cd but to Co and to Pb had the highest impact on lymphocyte function. Cd itself did not explain depression of lymphocyte function in multivariate analysis. Correlations in univariate analysis must be interpreted with caution. Comparison of external and internal exposure can explain this discrepancy to the original expectation: levels of Co in blood or in urine correlate with concentrations of Co in the air of the working place. Similar correlations were observed for Pb. However, no correlation between concentrations of Cd in blood or in urine with Cd air concentrations was observed. The lack of correlation for Cd cannot be explained by too low exposure to Cd in air, since 14 % of air measurements exceeded the current TLV for Cd. However, concentrations of Cd in body fluids were all below current TLV and ranged in concentrations commonly found in individuals without occupational exposure to Cd, comparable with Cd levels in smokers. On the other hand cigarette smoking increased Cd concentration in blood and urine. This discrepancy can be explained by the preferential occupational use of Cd salts with low solubility in the population under investigation. Thus, the associations of Cd in air and lymphocyte function should be regarded as a secondary effect due to the correlation of Cd and Co in air. However, the correlation of Cd in urine and lymphocyte function, as detected in our study in univariate analysis suggests that Cd principally is able to suppress immune function.

An interesting scenario was observed with respect to iron concentration in serum. A strong trend to lower concentrations of Pb, Co and Cd in body fluids was observed in individuals with higher iron serum concentrations. Most probably, this phenomenon can be explained by inhibition of intestinal heavy metal resorption due to high iron serum levels (Groten et al., 1991). This mechanism could also explain, why exposed individuals with high iron serum levels have higher rates of lymphocyte proliferation and a higher degree of *in vitro*-chromate resistance (Feron et al., 1995). Thus, iron in serum might be able to influence susceptibility to immunotoxic effects of heavy metals.

Corresponding investigations on this cohort revealed an increase in DNA-damage measured by DNA single strand break analysis and an impaired DNA repair capacity (Hengstler et al., 2003). These results shed some light on the underlying mechanisms of immunotoxicity observed in these persons. Cd, Co and Cr(VI) are capable to induce DNA strand breaks (Dally et al., 1997; Beyersmann et al., 1992). At least one cause of these lesions is the production of oxygen radicals (Kasprzak et al., 1994). The exposure to Pb, as well, has been shown to be correlated to oxidative DNA damage (Avery et al., 1996; Schilderman et al., 1997). Moreover, DNA repair is inhibited by Co, Cd and Pb (Dally et al., 1997; Beyersmann et al., 1997; Hartwig et al, 1990). All these effects can lead to a cell cycle arrest, thus delaying the velocity of proliferation (Hengstler et al., 2003).

In conclusion, exposures to Co and Pb in concentrations below or close to the current TLV lead to clear immunotoxic effects. A synergistic effect could be shown. Besides the conventional lymphocyte proliferation assay the newly established chromate resistance assay improves identification of immunotoxic exposures.

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