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THE EFFECT OF HEAVY METALS ON THE IMMUNE SYSTEM AT LOW CONCENTRATIONS

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Abstract. The present study describes the effect of cadmium on lymphokines that cannot be directly traced to an allergen, or antigen in order to be able to explain various immunological processes.
Exposure to various environmental pollutants is known to induce epithelial and inflammatory changes, characterized by a release of cytokines and other soluble mediators. Heavy metals like $CdCl_2$ can induce, or inhibit the synthesis and expression of the inflammatory cytokines IL-1β, IL-4, IL-6, TNF-α, IFN-γ and ICAM-1. Normal human peripherial blood mononuclear cells (PBMCs) were exposed for different periods of times (1 to 96 h) to 0, 5, 25 and 50 μmoles $CdCl_2$, and mRNA for the above cytokines was quantified by RT-PCR.
Highly purified blood B cells and PBMCs from healthy donors were stimulated with IL-4 and aCD40 mAb and incubated with non-toxic concentrations of cadmium chloride (0,1–10 μmol). Levels of IgG and IgE were measured in the supernatants. Proliferation and expression of surface markers were determined by measuring [3H]-thymidine incorporation and by flow cytometry.
The study showed that the *in vitro* synthesis of IgE by purified B cells or PBMCs stimulated with IL-4/aCD40 is inhibited by Cd at doses as low as 0,1 μM. Cd was found to inhibit IL-4/aCD40 induced proliferation of purified B cells and PBMCs in a dose dependent fashion. Most strikingly, only IgE but not IgG synthesis of purified B cells was inhibited by Cd.
These data suggest that inhibition of IgE synthesis in human B lymphocytes by Cd seems to be a selective effect on immunoglobulin synthesis.

Key words:
Cadmium, Lymphokines, IgG, IgE, hsp70, Stimulation

INTRODUCTION

Many metals occur naturally in the environment in organic or inorganic compounds [1]. Various industrial and combustion processes release heavy metals into the ambient air, lead to depositions and thus become responsible for contamination of the soil. Heavy metals are mostly absorbed either via airways [2,3] or via intestines. Heavy metals absorbed via intestines, as far as they are fairly water-soluble, are transmitted to several organs through the circulatory system and are absorbed by different systems. This form of absorption is particularly significant to the overall heavy metal load in the human body as higher concentrations of these metals can be absorbed [4,5]. Already low concentrations of heavy metals are sufficient to affect the respiratory tract [2,3,6]. Different cells, such as endothelial cells, epithelial cells, and alveolar macrophages are directly affected [2].

The immune system is a complex system of cells with various functions which in turn are regulated by soluble glycoproteins, the lymphokines [2,7]. Lymphokines are produced by immunocompetent cells, lymphocytes and monocytes, but they are also secreted by endothelial and epithelial cells [1,3,8]. It is the task of the immune system to protect the body from bacteria, viruses, parasites and fungi,

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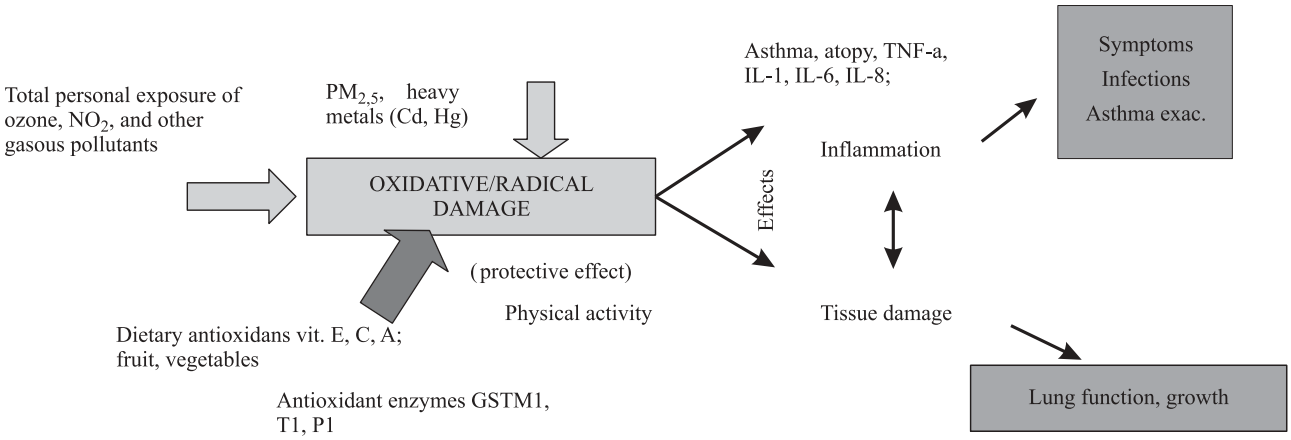

Fig. 1. Biologic impact pathways of various solid and gaseous pollutants, or particulate on acute and chronic respiratory effects and risk factors. Pollutants are able to trigger distinct immunological processes or intensify existing ones.

but also to destroy malignantly transformed cells or virus-producing cells. The harmonious interplay of the various cells and the targetted regulation of the cells through the production of lymphokines guarantees that the defense mechanism of the system does not degenerate [9].

Given the complexity and the variety of the immune system, there are many points of attack for heavy metals in order to influence the function of various cells, either by inhibition or stimulation, leading to a chain reaction and pathological response [4,10–24]. Although cadmium (Cd) normally occurs only in low concentrations in the ambient air, it is assumed, given its toxic potency, that it significantly influences the respiratory system and especially the immune system of airways [2]. Thus it may play a crucial role in the pathogenesis of respiratory tract infections. Gardner et al. [13] have demonstrated an increased susceptibility of mice to *S.zooepidemicus* following Cd exposure to 0.1–1.6 mg/m^3 for 2 h. Also, an increased mortality due to bacteria, as well as impaired bacterial clearance, and a decrease of alveolar macrophages (AM) in the bronchoalveolar lavage fluid, following an exposure to $CdCl_2$, have been reported. Whereas, exposure to airborne Cd led to a decreased defense against bacterial infection. Different reactions to viruses have been described in connection with Cd absorption. Thus immunotoxicity may have different consequences, depending on the concentration and the physico-chemical behavior of heavy metals. Immunotoxicity can be subdivided into direct immunotoxicity, hypersensitivity and autoimmunity [25–33].

Direct immunotoxicity

The main focus of this paper is the direct immuntoxicity, which in turn can be subdivided into immunosuppression and immunostimulation. Epidemiological studies of the past 20 years have shown that allergical diseases have greatly increased, especially in urban areas [3,6]. This is due to a number of reasons one of them is the changed quality of pollutants (Fig. 1) [3,34,35].

So far, little is known about the combined effect of pollutants, and also about the interaction of biological substances, like viruses, bacteria and their products, such as endotoxins and heavy metals [36]. Several animal experiments have been designed to clarify the complex reactions which lead to an increased inflammation or infections. Several *in-vitro* models deal with this problem [1,8,15,18,19].

Influence on cells

Heavy metals and their salts may influence cells in two ways:

1) by penetrating the interior of the cell through calcium channels of the L-type; and

2) by reacting with surface structures of the cell (see Fig. 5).

The activity of calcium channels is regulated by a wide variety of intracellular signaling pathways. Probably the three best understood are: (i) binding and activation of calmodulin, (ii) phosphorylation by several protein kinases, and (iii) binding of G protein βn subunits. These pathways not only modulate calcium channel activity but they are themselves modulated in various ways by calcium influx through calcium channels, and many interact with one another. Thus, complex feedback mechanisms among calcium channels and intracellular signaling exist, and are likely the targets for both environmental and genetic disorders. Calcium channels play key, but far from exclusive, roles in regulating intracellular calcium homeostasis. Both pathologic conditions and toxicologic insult (e.g. by methylmercury, lead or other metals) produce alterations in calcium homeostasis that may lead to cell death.

MATERIALS AND METHODS

Blood collection and analysis

Blood of 9 healthy adult donors (4 males and 5 females) was collected into heparinized tubes (VACUETTE; Greiner Labortechnik GmbH, Kremsmünster, Austria) and immediately processed. For each blood sample, the total and differential white blood cell counts were obtained using CELL-DYN 3500 (Abbott Laboratories, CA, USA). Peripheral blood mononuclear cells (PBMCs) were isolatated and the relative amounts of monocytes and lymphocytes in the PBMCs preparations were determined using two-color flow-cytometric analysis (PAS flow cytometer; Partec, Münster, Germany).

Monocyte counts measured in whole blood ranged from 7.1 to 11.3% (median value 9.1%). In PBMCs preparations, the relative proportion of monocytes was remarkably higher, ranging from 17.1 to 24.3% (median value, 20.9%). Variation between donors was ≤22%.

Cell culture and exposure

Peripheral blood mononuclear cells were isolated from heparinized venous blood by Ficoll gradient centrifugation (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Vienna, Austria) as described in detail elsewhere [37]. Mononuclear cells were suspended at a density of $1.5 \cdot 10^6$ cells/ml in RPMI 1640 (Life Technologies, Vienna, Austria), supplemented with 10% FCS (PAA Laboratories, Linz, Austria), 2mM L-glutamine and 25 mM HEPES (Life Technologies) without addition of antibiotics. Two ml of the PBMCs suspension were transferred to each well of 24-well suspension culture plates (Greiner, Kremsmünster, Austria). Cell culture was carried out at 37°C in a humidified 95% air/5% CO_2 athmosphere. PBMCs were cultured for 48 h before cadmium exposure. Cadmium chloride (Sigma-Aldrich, Vienna, Austria) was dissolved in Milli-Q purified water (Millipore Corporation, Bedford, USA) at 100 mM and filter-sterilized through 0.2 μm filters (Sartorius, Göttingen, Germany). Only endotoxin-free solutions were used. Stock solutions were further diluted in serum-free culture medium to a concentration of 5 mmol/l, and aliquots of this working solution were added to the cultures to produce final concentrations of 5, 25, and 50 μmol/l. After the desired exposure time, the cells were harvested by centrifugation and the supernatants were collected and stored at -70°C. Pelleted cells were washed twice with cold PBS (Life Technologies) and stored at -70°C until further use. All incubations were done in duplicate.

Isolation of B-lymphocytes

Purified B cells were isolated by a negative B cell isolation kit from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Briefly, PBMCs (1.10^8/ml) were incubated with FcR blocking reagent (1:5 final dilution) and a hapten-conjugated antibody-cocktail (1:6.5 final dilution) in cold PBS supplemented with 0.5% BSA, 2mM EDTA and 10% autologous serum for 10 min at 7–8°C. After washing cells twice, anti-hapten antibody-coupled microbeads (1:5 final dilution) were added for another incubation of 15 min at 7–8°C. Finally, 500 μl washed cell suspension was loaded onto a separation column (MACS LS^+, Miltenyi Biotec) in the magnetic field of a high energy permanent magnet, and B cells were eluted by rinsing the column with cold PBS/0.5% BSA/2mM EDTA/10% autologous serum. Remaining erythrocytes were removed by hypo-

tonic lysis. The B cell preparation routinely was at least 95% pure ($CD19^+$), with less than 0.5% $CD3^+$ (T cells) and $CD14^+$ (monocytes), respectively, as shown by flow cytometry. Cell viability, as assessed by trypan blue exclusion, was always >99%.

Measurements of cadmium cytotoxicity

Cell membrane integrity. Measurement of lactate dehydrogenase (LDH) leakage into the medium was used as a marker of cell membrane lysis. Freshly isolated PBMCs were seeded at a density of $1.5 \cdot 10^6$ cells/ml into 24-well plates in triplicate and cultured for 48 h. Then, $CdCl_2$ was added to the wells at final concentrations ranging from 5 to 50 μmol/l. After an incubation of 24 and 48 h with cadmium, culture supernatants were assayed for the presence of LDH using a commercial kit (Promega, Mannheim, Germany).

Metabolic activity assay. The metabolic activity of cells was measured using a colorimetric assay based on the cleavage of the tetrazolium-salt WST-1 (Boehringer Mannheim, Germany) to formazan by mitochondrial dehydrogenases in viable cells. Formazan dye formation, which can be photometrically determined, was used as a parameter reflecting the cellular metabolic activity. Cell incubations (in triplicate) were done under conditions similar to the LDH leakage assay. Absorbance at 450–620 nm was measured using an Anthos htIII (Fresenius GmbH, Linz, Austria) microplate reader.

RT-PCR assay

We have developed a highly reproducible RT-PCR assay to analyze profiles of mRNA expression in human PBMCs. An extensive validation of the method has been previously published [19,37–39].

Cellular total RNA was extracted using the Quiagen RNeasy™ system (Quiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RNA samples were quantified spectrophotometrically at 260/280 nm and stored at -70°C until use.

DNAse treatment of RNA samples. Genes encoding stress-inducible heat shock proteins do not contain introns. Therefore, RNA samples were treated with RNase-free DNAse (Promega) prior to reverse transcription to avoid amplification of contaminating genomic DNA; 1.5 μg of total RNA, 3 μl of 5x First Strand Buffer (250 mM Tris HCl, pH 8.3), 375 mM KCl, 15 mM $MgCl_2$ (Life Technologies); 1 U of RNAse-free DNAse (Promega) and DEPC-treated water were combined in a final reaction volume of 15 μl. After incubation at 37°C for 15 min, the reaction was stopped by adding 1.5 μl of 25 mM EDTA (Life Technologies). The samples were heated to 75°C for 10 min and kept on ice until further processing.

First strand cDNA synthesis. 13.5 μl of a master mix containing 3 μl of First Strand Buffer (Life Technologies), 3 μl of 0.1M DTT (Life Technologies), 0.6 μl of 25 mM dNTP-mix (Pharmacia), 15 U RNAguard (Pharmacia), 1 μl of 100 μM random hexamer primers (Pharmacia), 200 U M-MLV reverse transcriptase (Life Technologies) and DEPC-treated water were added to each tube. Reverse transcription was carried out in a total reaction volume of 30 μl at 37°C for 60 min, followed by inactivation of the reverse transcriptase at 75°C for 10 min.

Polymerase chain reaction. Gene-specific primer pairs were selected according to sequences published in GenBank human DNA database. Synthesis and HPLC-purification of oligonucleotide primers was performed by MWG-Biotech (Ebersberg, Germany). PCR amplification of specific cDNA sequences within the reverse transcription mixture was performed in parallel reactions. Aliquots of the reverse transcription reaction (2.5 μl for β-actin, L30, hsp70, IL-8, and IL-1ß; 4.0 μl for IL-1α, IL-6, and TNF-α) were combined with 5 μl of 10x PCR buffer (1x is 10 mM Tris-HCl, pH 8.8 at 25° C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100) (Finnzymes Oy, Finland), 0.4 μl of 25 mM dNTP-mix (Pharmacia), 12.5 pmol each of the primer pair specific for the target cDNA to be amplified, 1.0 U of DynaZyme™ (Finnzymes) and water in a final reaction volume of 50 μl. The reaction mixtures were overlaid with mineral oil (Sigma-Aldrich) and PCR amplification was done in a thermal cycler (TRIO Thermobloc; Biometra, Germany). Following an initial denaturation step at 95°C for 2.5 min, amplifications were cycled for 1 min at 95°C, 30 sec at 60°C (59°C for hsp70), and 30 sec at 72°C. The last cycle was followed by a final

Table 1. PCR primers, cycle number, and sizes of amplified PCR products

Amplified RNA	Primer sequence (5´–3´)	Cycle number	Product (bp)
hsp70	ATCGACCTGGCACCACCTACAGCACCATGGAGATCT	25	348
β-actin	GCAAGAGAGGCATCCTCACCGCACAGCCTGGATAGCAACG	26	240
l30	GAAGTACGTCCTGGGGTACAAGTCAGAGTCACCTGGATCAAT	23	236
IL-1α	GTCTCTGAATCAGAAATCCTTCTATCCATGTCAAATTTCACTGCTTCATCC	33	421
IL-1β	AAACAGATGAAGTGCTCCTFCAGGTGGAGAACACCACTTGTTGCTCCA	29	391
TNF-α	CAGAGGGAAGAGTTCCCCAGCCTTGGTCTGGTAGGAGACG	33	325
IL-8	CTCTTGGCAGCCTTCCTGATTCAACCCTCTGCACCCAGTTTTCC	26	240
IL-6	CAGCCACTCACCTCTTCAGAACTGCAGGAACTGGATCAGGAC	28	332

extension step at 72°C for 7 min. To assure that RT-PCT reactions were within the linear range of PCR, cycle titrations were performed for each distinct target cDNA. The optimum cycle numbers determined empirically in preliminary amplifications are listed in Table 1.

The PCR products (5–10 μl) were separated by electrophoresis through 2% agarose (Sigma) at 80 V for 60 min in Tris acetate/EDTA buffer containing 0.5 μg/ml ethidium bromide. The relative amounts of mRNA were determined visually by densitometric scanning of ethidium bromide-stained gels (Gel-Doc 1000; BioRad, USA). Only amplification products of the size predicted by the mature mRNA sequence were quantified. Gels were discarded when inappropriate large amplification products were present. To detect unwanted amplification of genomic DNA contaminating the RNA preparations, negative control reactions (no enzyme added during the reverse transcription step) were included in all experimental series.

Determination of IgE

Determination of IgE concentrations was done by a fluoroimmunoassay (FIA) (0.35–100 U/ml) with the Pharmacia ImmunoCAP system (UniCAP 100; Pharmacia, Uppsala, Sweden). Results reflect means of duplicate determinations. The presence of Cd did not interfere with the test system.

Determination of IgG

Determination of IgG concentrations was done by ELISA using a commercial antibody-pair from Bethyl Lab. Inc. (USA) according to the manufacturer´s recommendations. Briefly, microtiter plates (Nunc A/S, Denmark) were coated with goat anti-human IgG-Fc antibody (1:100 final dilution in 0.05 M sodium carbonate, pH 9.6) overnight at 4°C. After blocking with 0.05 M Tris-Cl pH 8.0/ 0.15 M NaCl containing 1% BSA for 30 min at room temperature (RT), test samples and serially diluted IgG standards were added to the wells in duplicate and incubated for 1 h at RT. After washing, horseradish peroxidase-conjugated goat anti-human IgG-Fc antibody (1:100.000 final dilution) was added and incubated for 1 h at RT. TMB (3,3´,5,5´-tetramethylbenzidine)/H_2O_2 solution was used as substrate and the optical density was measured at 450 nm with a 96 well-plate reader (Anthos, Austria). The lower limit of sensitivity of this assay was 7.8 ng/ml.

Statistical analysis

Analyses were performed using the SPSS software package (SPSS Inc., Chicago, USA). Experimental data were tested for normal distribution (Kolmogorov-Smirnov test). Data derived from cytotoxicity assays represent means ± SD. All other data are presented as the median with range. Differences between cadmium-exposed cultures and untreated controls were analyzed using the Mann-Whitney U test and Wilcoxon signed rank test, respectively; $p < 0.05$ was regarded as statistically significant.

The release of cytokines was evaluated, using a univariate two-way ANOVA model with one repeated measure (time or Cd-concentration). The overall effects of time or

Cd-concentration were considered significant if $p < 0.05$. Due to unequal sample sizes among groups, Bonferroni multiple pair wise t-tests with overall $\alpha = 0.05$ was applied. After determining the best-fit values of the variables in the equation, Prism (GraphPad Software, Inc. Oberlin Drive, San Diego, USA) calculates and plots the best-fit curve.

RESULTS

PBMC-based *in vitro* system

Human PBMCs were selected as an *in vitro* model to investigate effects of low-level cadmium exposure on the human immune system. Several important aspects should be taken into consideration when using PBMCs for *in vitro* gene expression studies. First, the well-known phenomenon of "monocyte adherence": freshly isolated monocytes spontaneously adhere to plastic or glass surfaces, which is accompanied by a transiently stimulated expression of several genes, e.g. cytokines [40]. Second, bacterial endotoxins as potent inducers of proinflammatory cytokines in human PBMCs. Third, recently demonstrated finding that collagenase and Ficoll are potent inducers of IL-1ß generation in human PBMCs [41]. Fourth, the report that the synthesis of proinflammatory cytokines may also be triggered by hyperosmotic culture conditions. Compared to human plasma (280–295 mOsM), routine tissue culture medium is hyperosmotic (305 mOsm) [42].

For gene expression studies, a "quiescent" status of the cells is clearly a major experimental prerequisite. Specific effects of test compounds may be masked when experiments are performed immediately after the isolation procedure, or shortly after cell seeding (unpublished personal observations). Moreover, it cannot be ruled out that non-specific activation of the cells may render them refractory to experimental stimulation for prolonged time periods.

To eliminate such interferences, we have developed a cell system which uses PBMCs that have been incubated in a standardized cell culture system for at least 48 h after cell preparation. As it was demonstrated in our recent study [37], this "lag phase" allows the cells to recover from the isolation procedures and to restore regular mRNA expression, thus providing a reproducible basis for experimental *in vitro* exposures.

Analysis of cadmium cytotoxicity

PBMCs were cultured in the presence of 0, 5, 25, and 50 μmol/l $CdCl_2$ (final concentrations) for 24 h and 48 h, respectively. Direct effects of the heavy metal upon cultures were monitored via (a) release of lactate dehydrogenase (LDH) into the culture supernatant, indicating damage of plasma membranes, and (b) specific activity of mitochondrial dehydrogenases as a measure of the metabolic activity of viable cells.

A direct toxic effect of $CdCl_2$ was observed at concentrations higher than 75 μmol/l. At lower concentrations (5 to 50 μmol/l) of $CdCl_2$, direct cytotoxic cadmium effects were not detected. Based on the results of these experiments, $CdCl_2$ concentrations up to 50 μmol/l were defined as the "non-cytotoxic" range in the selected cell model.

Analysis of mRNA expression

In a first set of experiments, PBMCs were cultured in the presence of non-cytotoxic doses of $CdCl_2$ (5, 25, and 50 μmol/l, final concentrations) to examine the time course of mRNA expression. Short-term cadmium effects were measured in cells after 1, 3, 6, and 24 h of exposure, respectively. Cells were also exposed for 48 h in order to assess whether some cadmium effects might occur later in the culture.

As a general feature, changes in mRNA expression pattern occurred very rapidly after confrontation with the heavy metal. For most of the genes analyzed here, major alterations in mRNA levels were restricted to a period of a few hours immediately after the addition of the metal solutions. In all cases, basal mRNA expression was restored in the cells after 24 h and remained unaffected during subsequent exposure. Based on these observations, a one-hour exposure time was chosen for further detailed analysis of the cadmium effects.

To estimate the limits of the selected cell model, cultures were also treated with cytotoxic doses of 100 μmol/l $CdCl_2$ for 1 and 4 h.

hsp70

Cadmium is well-known as a highly potent inducer of heat shock proteins in mammalian cells [39,43]. Therefore, we have measured hsp70 mRNA expression as a positive control to demonstrate that cellular responsiveness was fully preserved under the selected experimental conditions [39,43–49].

Time-course studies revealed that confrontation with non-cytotoxic cadmium concentrations caused a transient accumulation of hsp70 mRNA in PBMCs. In the presence of 50 μmol/l of $CdCl_2$, hsp70 mRNA levels steadily increased up to 6 h of exposure, whereas in cultures exposed to 5 and 25 μmol/l the expression levels peaked within 1 h, rapidly decreasing thereafter. $CdCl_2$ concentrations below 5 μmol/l failed to induce the specific hsp70 response in the cell system.

The hsp70 mRNA expression pattern was further characterized in one-h-exposures. Accumulation of hsp70 mRNA was clearly dose-dependent, with a strong linear relation ($r = 0.7344$; $p < 0.0001$) over the non-cytotoxic range (0 to 50 μmol/l $CdCl_2$) (Fig. 2).

Housekeeping genes (β-actin, ribosomal protein L30)

For a quantitative analysis of mRNA expression, comparisons between samples can be made by relating gene expression levels to housekeeping gene expression; the latter regarded as not being affected by exposure conditions. However, exposure effects on "housekeeping" genes do occur. Therefore, we have examined the impact of cadmium exposure on the expression levels of two genes which are both regarded as indicators of constitutive gene expression, i.e. β-actin (a major constituent of cytoskeleton) and L30 (ribosomal protein).

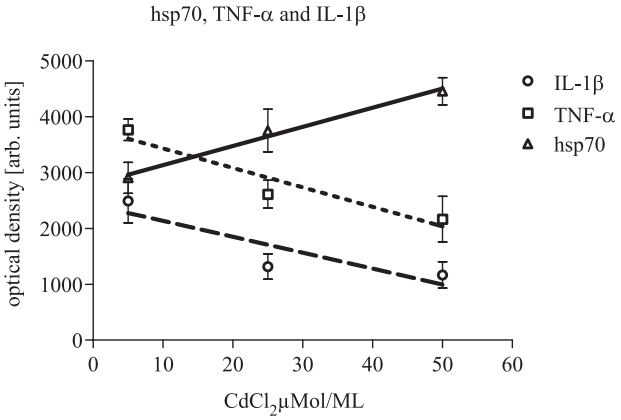

Fig. 2. Hsp70 increases linearly with the concentration of cadmium. An inverse correlation originates between the behavior of hsp70 and cyrokines IL-1β and TNF-α.

Similar cadmium effects were observed for both genes. Steady-state mRNA expression was not affected by 5 μmol/l of $CdCl_2$, but a tendency of progressively declining levels was noticed over the ascending scale of cadmium concentrations. This trend was slightly more pronounced in the case of β-actin, since one-h-exposure to 50 μmol/l of $CdCl_2$ led to about 16% reduction ($p < 0.02$), whereas L30 mRNA was only marginally decreased.

Anyhow, the steady state mRNA expression of both housekeeping genes was dramatically reduced in cultures exposed to concentrations of 100 μmol/l of $CdCl_2$. The data clearly indicate that mRNA expression of housekeeping genes as a reference can only be used under non-cytotoxic experimental conditions.

In spite of this, normalization of cytokine mRNA levels to either one of the two housekeepers did not change the results. Therefore, mRNA data of cytokine genes (see below) represent signal densities of cDNA from ethidium bromide-stained agarose gels, without correlation with signals from control genes.

Cytokines

Exposure to non-cytotoxic doses of $CdCl_2$ rapidly induced accumulation of cytokine mRNAs. In the time course of the studies performed in advance, cytokines appeared almost simultaneously. The mRNA levels generally raised within 30 min, peaked around 1 to 3 h, and decreased again within 6 h. After 24 h, basal mRNA expression was restored and remained unchanged thereafter.

In all cases, the highest accumulation of mRNA was found in cells exposed to 5 μmol/l of $CdCl_2$. Within 1 h, 5–6 fold increase in mRNA levels over control for IL-1α, IL-1β and IL-8, about 3–9 fold for IL-6, and 1.6-fold for TNF-α were observed. The large dispersion of data between distinct blood donors (coefficient of variation ≤68%) indicated high-grade inter-individual variability in cytokine responses. Endotoxin is a strong stimulator of

cytokine response. To neutralize possible endotoxin contaminations in the $CdCl_2$ test solutions, we performed control experiments, using the LPS-binding substance, polymyxin B sulphate (Sigma). Pretreatment of cultures with 10 μg/ml polymyxin B for 30 min prior to cadmium exposure did not inhibit the accumulation of cytokine mRNAs, indicating that the effect was not caused by endotoxin contamination.

With increasing cadmium concentrations, accumulation of IL-1α, IL-1ß, and TNF-α mRNAs was progressively depressed. Accumulation rates were significantly lower in PBMCs cultured in the presence of 25 or 50 μmol/l of $CdCl_2$ as compared to 5 μmol/l. The dose-related depression was particularly pronounced in the case of TNF-α, where mRNA accumulation was found only in cultures exposed to the lowest dose (of 5 μmol/l), but not at higher Cd concentrations. Exposure to cytotoxic cadmium concentration of 100 μmol/l abrogated accumulation of all cytokine mRNAs within 1 h, and resulted in a pronounced reduction of up to 70% in steady-state mRNA expression after 4 h (Fig. 2).

By contrast, for IL-8 and IL-6 the depressive effects of increasing Cd concentrations were not so strongly pronounced. The accumulation rates of both mRNA types were similar over the non-cytotoxic range of $CdCl_2$. Only in the presence of 100 μmol/l of $CdCl_2$, a significant fall in mRNA accumulation was found, although the mRNA levels of both cytokines were always remarkably higher than in untreated control cultures (3.4 fold for IL-8, and 2.5 fold for IL-6, respectively; $p < 0.02$). Even after a 4-h exposure, the levels of both mRNA species were still twice as high as in the control ($p < 0.02$) (Table 2).

Table 2. Genes proven by PCR. Some genes are influenced by the burden of cadmium (+), others are up-regulated more intensively

Gene	5	25	50
	μM $CdCl_2$		
Hsp70	+	++	+++
TNF-	+++	++	+
IL-1	+++	++	+
IL-4	+	+	+
IL-6	+++	+++	+++
IL-8	+++	+++	+++
INF-	n.s.	n.s.	++

IgE concentration significantly decreases with increasing Cd concentration. This is a non-linear regression and the equation represents a one site binding (hyperbola).

Through aCD40/IL4 stimulation, mostly those cells are activated which are able to produce IgE. Normally neither cadmium nor aCD40/IL4 stimulation can stimulate B-lymphocytes to produce IgG. Stimulation permits merely to detect IgG traces. As a result of combining aCD40/IL4 stimulation and cadmium loads, the cells started to produce measurable quantities of IgG. Whereas IgG production was close to the limit of detection at low Cd concentrations, IgG concentration was clearly measurable in the supernatant by increasing Cd concentrations. This, too, is a non-linear correlation; the equation is polynomal second order (Fig. 3). The concentration of immunoglobulins

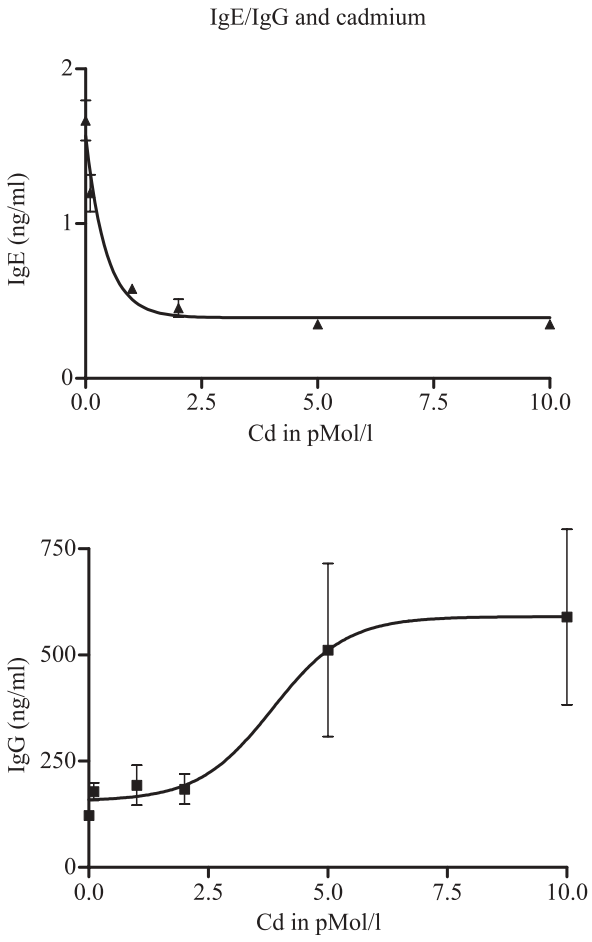

Fig. 3. An inverse effect of release of IgG and IgE in context of the increased cadmium concentration can be observed. While the amount of IgE decreases with increasing Cd concentrations, the concentration of IgG also increases.

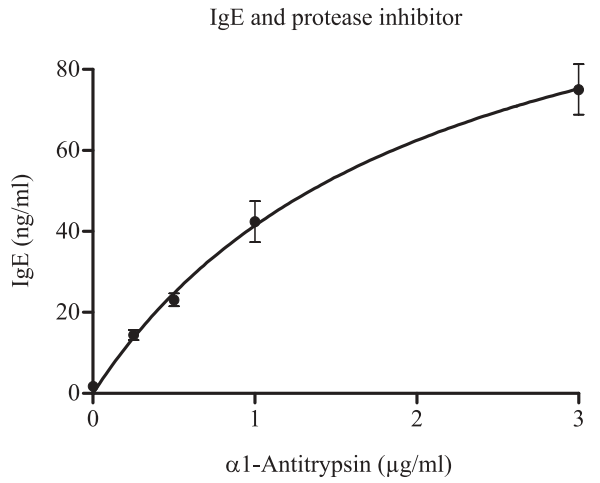

Fig. 4. The non-linear relation between PI concentration and IgE release. Equation: One site binding (hyperbola) $Y = B_{max} \bullet X/(Kd + X)$. This describes the binding of a ligand to a receptor that follows the law of mass action. B_{max} is the maximum binding, and Kd is the concentration of ligand required to reach half-maximum binding ($R^2 = 0.9573$).

in the supernatant was determined by ELISA. The limit of detection was 50 ng/ml for IgG and 0.35 ng/ml for IgE.

If B-cells are incubated with a protease-inhibitor (PI), the released amount of IgE increases with the concentration of the added quantity of PI. A non-linear dependence between increasing PI concentration and IgE synthesis has been observed ($p \leq 0.0001$). Now IgE is produced up to the tenfold quantity (Fig. 4).

DISCUSSION

Heavy metals influence the complex immune system at different levels, always affecting its cells. If metals at low concentrations encounter proinflammatory cells, they may stimulate the production of proteins – cytokines. Cytokines (IL4, IL5, IL6, IL8, TNF-α) of the proinflammatory cells stimulate other B- or T-type lymphocytes, and those of TH2-cell line predominate at low Cd concentration. However, at higher concentrations of heavy metals, the realease of cytokines of TH1-cell line predominates, which means that an immunosuppression of the antibody-production is probable at higher Cd concentrations. In short, at low Cd concentration this results in an activation of parts of the immune system, but the target against which the system is directed is lacking. An excessive reaction that occurs may trigger paradoxical phenomena, manifesting itself as an obstruction of the respiratory tract. As not only lymphocytes or monocytes but also endothelial and epithelial cells possess lymphokine receptors, we may postulate that heavy metals, such as cadmium may well induce inflammatory reactions by producing lymphokines. This, however, requires very low Cd concentrations which can be transported into the respiratory tract through particles.

Cytokine expression in cadmium exposed PBMCs was modulated in a highly differential fashion. Low, non-cytotoxic metal doses induced a transient expression of inflammatory cytokines, as indicated by an accumulation of their mRNAs. Maximum stimulation was found in the presence of the lowest metal dose (5 μM of $CdCI_2$), whereas in the presence of higher Cd concentrations (>25 μM) mRNA accumulation was progressively reduced. Interestingly, dose-related suppression was only observed for IL-lα, IL-lß, and TNF-α, but not for IL-6 and IL-8 mRNA, and this shift in cytokine expression favouring IL-6 and IL-8 was particularly striking under conditions, which must be considered as highly cytotoxic. The mRNA levels of both IL-6 and IL-8 were still twice as high as in the controls even in the presence of a cytotoxic cadmium concentration (100 μM of $CdCI_2$), when steady-state mRNA expression was generally reduced to a minimum (including genes that are regarded as constitutively expressed, e.g. ß-actin and ribosomal protein L30).

The molecular basis for stimulated cytokine expression in Cd exposed PBMCs remains still unknown. It is a striking feature of cadmium to specifically induce the transcription of several classes of genes, including genes involved in immunity and inflammation [50]. However, the intermediate events between Cd contact and induction of cytokine gene expression are not completely defined and may involve numerous pathways. A highly potential candidate for a primary target affected by Cd action is the Ca^{2+} signaling system [51]. Cadmium provokes immediate changes in cytosolic free calcium concentrations [Ca^{2+}] in various mammalian cells, which is of particular significance since it increases in the intracellular calcium

concentration [Ca^{2+}] and controls a diverse range of cell functions, including cytokine production.

Cadmium also strongly promotes the expression of heat shock proteins in various biological models [52]. Heat shock proteins have been proposed as general markers of cellular aggression and their use in environmental monitoring is often suggested [43]. Stress proteins and stress-related factors fulfil important regulatory functions in the expression of pro-inflammatory genes [53]. Expression of hsp70 (a major heat shock protein) concomitantly inhibits production of IL-1 and TNF-α in monocytes and macrophages [54–56]. Both IL-1 and TNF-α are of highly inflammatory nature and produce fever, inflammation, tissue destruction and, even, shock and death [57]. Therefore, an abrogation of cytokine release caused by inducible hsp70 expression is thought to have protective effects against systemic inflammation [58].

In cadmium exposed PBMCs, hsp70 mRNA accumulation increased over the tested dose-range (between 5 and 50 μM of $CdCI_2$) with maximum steady-state levels at the highest dose. As already mentioned, heat shock induction was accompanied by decreasing levels of IL-1 and TNF-α mRNA, whereas accumulation of IL-6 and IL-8 mRNA was not reduced. No clear concept of the impact of heat shock response on IL-6 and IL-8 expression has been as yet defined. Depending on the experimental system, both depression and enhancement have been reported [59,60], but the question remains to be clarified.

As already mentioned, heavy metals can interact with surface structures. This reaction may be explained using the example of IgE synthesis.

IgE synthesis requires activation of 3 signals at B-lymphocyte, with TH2 lymphocytes playing a significant supportive role. As a first signal, specific surface immunoglobulines must be activated by an antigen or an allergen. This reaction is significant for the specificity of the immunoglobuline to be produced. Additionally, CD40 receptor must be activated by CD40 ligands of TH2 cell starting the proliferation of B-lymphocyte. The third and very important reaction is the activation of IL-4 receptor complex by IL-4. This reaction is important, because it leads to an immunoglobulin switch from IgG/IgM to IgE. Its product is a specific IgE. By activating CD40 receptor by means of mAb (aCD40) and by adding IL-4, B-lymphocytes can be stimulated to produce unspecific IgE.

B-lymphocytes from 9 different probands were highly purified using a cell sorter. Through very low cadmium concentrations (0.1–10 μM $CdCl_2$) and stimulation in the aCD40/IL4 model, these B-lymphocytes were stimulated to synthesize non-specific IgE. The immunoglobulin-synthesis of B-cells was influenced decisively by the incubation of cells with $CdCl_2$. IgE release decreased distinctly with the increased concentration of IgG in a non-linear relation with the concentration of $CdCl_2$.

Surprising is the fact that in this situation a cell is stimulated towards a very specific reaction, namely IgE synthesis. Normally aCD40/IL-4 stimulation only produces traces of IgG. Cadmium changes the signal in a manner that the cell responds to the synthesis of a different protein as the cell was stimulated to produce a specific stimulus.

The health effects of cadmium, especially absorption through inhalation, must be considered. Dust and other particles function as carriers, and transport the metal to the area of the respiratory tract where they can directly encounter the immune system. The smaller particles, the more serious are the consequences.

In addition to the stimulatory qualities of the metals, we were also able to observe reactions with structures of the lymphocyte cell membrane. After all, cadmium influenced

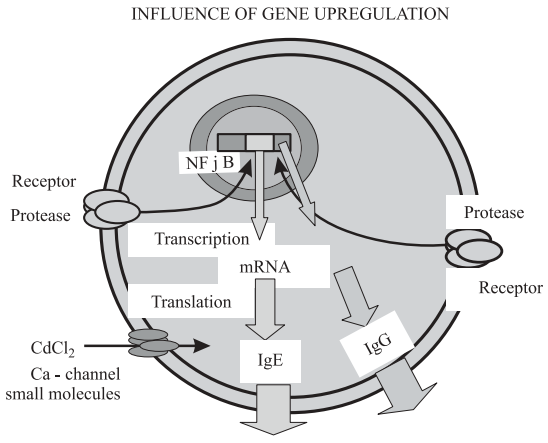

Fig. 5. Cadmium is able to influence B-lymphocytes by two ways: 1) by penetrating into the cell through Ca-channels, and 2) by interacting with structures on the surface of B-lymphocytes. There are observable, inhibitory and stimulating processes.

a signal of the cell in such a way that the cell was forced to produce a completely different protein than would have been induced by the original signal. This observation is particularly interesting from the point of view of molecular biology as it confirms the existence of much (all?) information on the cell genome that can be upregulated at a short notice through selective controls. To do this, very low Cd concentrations are required (Fig. 5).

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