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Effect of lead on the levels of some immunoregulatory cytokines in occupationally exposed workers

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Lead (Pb) may affect humoral and cellular immunity, acting on lymphocytes as well as on granulocytes and monocytes. Cytokines and nitric oxide (NO) play a central role in the immune balance. In this study, plasma levels of nitrates and nitrates (NOx), IL2, IL4, IL6, IL10, TNF-α and INF-γ, were measured in healthy workers with very low (Pb-B = 3.2–18.0 μg/dL) and low (Pb-B = 9.1–46.0 μg/dL) Pb-exposure compared to non-exposed workers. Low Pb-exposed workers (Pb-B = 9.1–46.0 μg/dL) were found to have significantly higher plasma IL-10 levels, and tendentially higher plasma TNF-α levels compared to non-exposed workers. This is the first report of a significant increase of plasma IL-10 levels in Pb-exposed workers. Plasma IL-10 increase was influenced by blood Pb levels even after correction for main confounding factors. No difference was found in plasma NOx levels between Pb-exposed and non-exposed workers, which is in agreement with previous findings exclusively regarding groups in the general population. Low Pb-exposure can induce an increase of pro-inflammatory cytokines, such as TNF-α, with a consequent increase of other cytokines, such as IL-10, considered a T cell cross-regulatory factor, suggesting possible interference of Pb in the system of immunophlogosis. Human & Experimental Toxicology (2007) 26, 551–556

Key words: cytokine; IL 10; lead; NOx; occupational exposure; TNF-α

Introduction

Lead (Pb) has the ability to alter the immune response even at moderate levels of exposure. 1,2 It is well established that this metal impairs humoral immune response, resulting in a decrease of antibody production. 3 Pb can also influence differentiation of B cells into antibody producing cells. 4 Moreover, it enhances production of serum IgE in humans. 5 Some studies have shown that Pb can reduce experimentally induced proliferation of animal and human lymphocytes. 6,7 However, according to other authors, suppressor T cells may represent the primary target for lead immunotoxicity. 8 Besides, it has been suggested that lead can also affect cells other than lymphocytes, such as granulocytes and monocytes. 2,9 The functional activities of these cells are actually regulated by a number of cytokines and by nitric oxide (NO), having both a central role in innate and adaptive immune responses.

It has recently been reported that production of NO and cytokines, such as interleukin-2 (IL-2), interleukin-10 (IL-10) and tumour necrosis factor-alpha (TNF-α), by splenocytes and adherent peritoneal cells in vitro is enhanced after incubation with lead. 10 However, in mouse adherent peritoneal cells and macrophages, an increased production of NO is recorded only at the lowest Pb in vitro concentrations, whereas an inhibitory effect is found at higher Pb concentration. 10,11

The aim of this study was to assess the effect of Pb exposure on plasma IL-2, interleukin-4 (IL-4), interleukin-6 (IL-6), IL-10, TNF-α, interferon gamma
(INF-γ) and N-oxides (NOx) levels in low-lead exposed workers compared to never exposed controls.

Materials and methods

Subjects
The study constituted 58 male workers with occupational exposure to lead (14 pottery and 44 foundry workers, aged 30–61 years), and 59 male workers of an alimentary plant (aged 25–61 years) who had never been exposed to this metal were controls. The research protocol was approved by the ethics committee of the Departments and submitted to the company and the workers’ representatives for agreement. Informed consent was obtained from all subjects prior to their inclusion in the study, according to the principles of the Declaration of Helsinki.

A questionnaire was administered to all the workers to obtain information on medical and occupational history, medications, smoking and drinking habits. Subjects who had not smoked for at least 1 year were considered non-smokers. Clinical data were obtained from the occupational health services of the three plants.

Collection and analysis of blood and urine samples
Venous blood (11 mL) was collected in the morning from each worker after a fast of at least 10 hours. Aliquots of 2.5 mL sodium heparinised venous blood were used to determine blood lead (Pb-B), zinc protoporphyrin (ZPP) and delta-aminolevulinic acid dehydratase (ALAD). Aliquots of 2.5 mL EDTA blood were used to measure red blood cell count (RBC), haematocrit (HTC), haemoglobin concentration (Hb), and white blood cell count (WBC). Sodium heparinised blood (6 mL) was centrifuged to obtain plasma, which was stored at −20°C until use for cytokine and NOx determinations.

Morning spot urine samples were collected in polypropylene containers. Urine (5 mL) was used to measure urinary lead (Pb-U), and to determine urinary delta-aminolevulinic acid (ALA-U).

Biological indicators of Pb exposure and effect on haeme synthesis
Pb-B concentrations were measured by a 5100ZL atomic absorption spectrophotometer (Perkin Elmer Italia, Monza, Italy) using the graphite furnace technique. In brief, heparinised blood was diluted with 0.2% Triton X-100 solution. Magnesium nitrate was used as matrix modifier. Diluted blood (20 μL) was injected into the furnace. Conditions were as recommended in the user manual. The absorbance was read at 283.3 nm with Zeeman-effect background correction. ZPP was determined in blood samples with a hematofluorometer (Hemafluor ZP, Buchler). ALAD was measured using the enzymatic colorimetric method. Pb-U was also measured in the atomic absorption spectrophotometer using the graphite furnace method. Urine was diluted 1:1 in distilled water and diluted urine (20 μL) was injected into the furnace. For ALA-U, we use a chromatographic spectrophotometric technique using an ion exchange resin column. Optic density was obtained at 553 nm in a Perkin Elmer Lambda 2 spectrophotometer.

Haematological parameters
All haematological tests were performed in a semi-automated Coulter Counter model JT using the Coulter Principle. RBC and MCV were measured by volume conductivity technology. Hb levels were read colorimetrically at 525 nm. Mean corpuscular volume (MCV) was determined from a histogram of the volume of the individual red cells as they were counted. HTC was automatically calculated from RBC and MCV using the formula: HTC = RBC (10^6/μL) * MCV(μL)/10.

Measurement of cytokine levels in plasma
Plasma levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ were measured using an in vitro enzyme-linked immunoabsorbent assay (ELISA), according to the kit’s instructions, which point coefficient variations intra and inter-assay lower than 10% (Pierce Endogen, USA), with some modifications. Briefly, samples were added to 96-microwell plates, precoated with a monoclonal antibody directed against each cytokine. Biotinylated antibody solutions were added and plates were incubated at 37°C as follows: 3 hours for IL-6, sensitivity limit 0.20 pg/mL; 2 hours for TNF-α, sensitivity limit 0.11 pg/mL; 6 hours for IL-4, sensitivity limit 0.6 pg/mL; 6 hours for IL-2, sensitivity limit 0.6 pg/mL; 12 hours for IFN-γ, sensitivity limit 0.10 pg/mL; 2 hours for IL-10, sensitivity limit 0.20 pg/mL. Incubation times for IL-2, IL-4 and IFN-γ were increased to obtain a lower sensitivity limit.

After incubation, plates were washed and a streptavidin-HRP solution was added; after a further wash and the addition of TMB-substrate solution, plates were incubated at 37°C for 30 min. 0.18 M sulphydric acid stopping solution was added, and absorbance at 450 nm was measured on a Wallac 1420 Victor 2 multilabel counter (EG&G Wallac, Finland). Concentrations were then calculated from a standard curve.
Experiments were carried out in duplicate, and values with a difference from the mean >10% were rejected. With preliminary experiments we found that accuracy of the measurement for each cytokine was B10%.

Plasma NOx

Plasma concentrations of nitrites and nitrates (NOx) were determined with a spectrophotometric assay using Greiss reaction-dependent generation of a chromophore.13,14 Briefly, plasma was centrifuged at 2000 rpm for 15 min at 4°C with ultrafilter Amicon cones. The eluate was treated with nitrate reductase enzyme (650 mU/mL), which converts all nitrate to nitrite. To this mixture (100 mL), an equal volume of Griess reagent (1% sulfanilamide/0.1 naphthyethyleneiamine dihydrochloride/25% H3PO4) was added, and incubated at room temperature for 10 min. The absorbance was read at 538 nm with the Wallac counter.

Samples were run in duplicate with blank and standard specimens. Concentrations of nitrites in the sample were read by extrapolating absorbance values from a standard curve obtained with NaNO2.

Statistical analysis

Data were summarised with the mean as a measure of central tendency, and standard deviation as a measure of dispersion. Differences between means were tested with one-way variance analysis (ANOVA). Comparisons between the means of variables showing a non-normal frequency distribution were performed after logarithmic transformation of measures. Chi-square test was performed to examine associations between categorical variables. Correlations between continuous variables were measured using Spearman’s rho non-parametric test. To verify the influence of some independent variables on the determination of a dependent variable, multiple stepwise regression models were used. The limit of significance was set at P < 0.05. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS version 12.0; SPSS, Chicago, IL, USA).

Results

Subjects

The main general characteristics and life habits of the three worker groups are shown in Table 1. No subject exhibited clinical signs or symptoms of infection at the time of the study; none was using drugs that could affect immunological assays. No clinically evident diseases were found in either exposed or non-exposed workers. No differences were found between mean values of RBC, HCT, MCV, Hb and WBC of the three worker groups (data not shown). No haematological abnormalities were detected in the workers examined.

Biological indicators of Pb exposure and effect on haeme synthesis

Table 2 shows that the mean levels of all Pb biological indicators are significantly different be-
between the three worker groups. Specifically, foundry workers had mean Pb-B levels significantly higher than pottery workers (Bonferroni test: \( P < 0.0001 \)). Therefore, these two groups of Pb-exposed workers will be referred to as low (foundry) and very low (pottery) Pb-exposed workers.

Very close correlations were found between Pb-B and other Pb biological indicators (data not shown).

**Cytokine profile**

The mean plasma IFN-\( \gamma \), IL-2, IL-4 and IL-6 levels were not significantly different between the three groups of workers in the study (Table 3). Mean plasma IL-10 and TNF-\( \alpha \) levels were significantly different between the three groups (Table 3). In particular, foundry workers had mean plasma IL-10 levels significantly higher than non-exposed workers (Bonferroni test: \( P < 0.05 \)), whereas it results only in a tendency toward higher plasma TNF-\( \alpha \) levels in foundry workers over the non-exposed controls (Bonferroni test: \( P = 0.056 \)). A slight, but significant, positive correlation was found between Pb-B and plasma IL-10 (Spearman test: rho 0.240, \( P < 0.05 \)) and TNF-\( \alpha \) (Spearman test: rho 0.214, \( P < 0.05 \)) levels in all 116 workers taken together. By using a multiple stepwise regression model in the whole population examined, plasma IL-10 was shown to be significantly influenced only by Pb-B level, even after correction for age, BMI, smoking (0/1) and alcohol consumption (0/1) (Model: \( F = 7.5; P < 0.01; R^2 = 0.249 \)). In a similar multiple stepwise regression model, Pb-B level was shown to influence plasma TNF-\( \alpha \) level, even after correction for age, BMI, smoking (0/1) and alcohol consumption (0/1) (Model: \( F = 6.7; P < 0.05; R^2 = 0.235 \)), in all 116 workers taken together.

**Concentration of NOx**

Mean NOx values were 23.73 ± 7.27 \( \mu \)M (range: 11.21–55.71) in controls, 28.44 ± 11.42 \( \mu \)M (15.23–57.65) in pottery workers, and 25.30 ± 8.70 \( \mu \)M (15.03–61.98) in foundry workers, respectively.

**Table 3** Cytokine plasma levels (mean ± standard deviation) (pg/mL) in controls and lead-exposed workers

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 59)</th>
<th>Pottery workers (n = 14)</th>
<th>Foundry workers (n = 43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-( \gamma )</td>
<td>0.74 ± 0.58</td>
<td>0.57 ± 0.53</td>
<td>0.57 ± 0.51</td>
</tr>
<tr>
<td>IL-2</td>
<td>3.34 ± 1.31</td>
<td>3.77 ± 1.94</td>
<td>3.92 ± 1.69</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.65 ± 0.84</td>
<td>0.50 ± 0.51</td>
<td>0.42 ± 0.43</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.73 ± 0.65</td>
<td>0.76 ± 0.51</td>
<td>0.81 ± 1.00</td>
</tr>
<tr>
<td>IL-10*</td>
<td>4.55 ± 3.89</td>
<td>4.68 ± 1.53</td>
<td>7.37 ± 8.00</td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>2.30 ± 1.39</td>
<td>3.66 ± 2.69</td>
<td>3.05 ± 1.66</td>
</tr>
</tbody>
</table>

\( \* P < 0.05 \) (ANOVA).

with no statistically significant difference between groups (\( F = 1.87; \text{n.s.} \)). No correlation was found between Pb-B and NOx level in all 116 workers taken together.

**Discussion**

In this study, low Pb-exposed workers (Pb-B = 9.1–46.0 \( \mu \)g/dL) were found to have significantly higher plasma IL-10 levels and tendentially higher plasma TNF-\( \alpha \) levels in comparison to non-exposed workers. Moreover, these two cytokines were significantly affected by Pb-B levels even after correction for the main confounding variables in all the workers in the study. No significant differences were found in plasma levels of other cytokines examined, and in NOx concentrations of these Pb-exposed and non-exposed workers.

This is the first report of a significant increase of plasma IL-10 levels in Pb-exposed workers with respect to non-exposed workers. Data from *in vitro* experimental studies are contrasting. Following Pb treatment, an enhanced production of IL-10 was found by mice spleen cells,10 and mice gut tissue tolerised with ovalumin,15 whereas decreased amounts of IL-10 were produced by mice macrophages.16 IL-10 is one of the cytokines involved in chronic inflammation, taking part both in humoral and cellular immune mechanisms.17 It is produced by CD4+ T cells, activated CD8+ cells, and activated B cells,18 leading also to a reduction of antigen-specific T cells proliferation and inhibition of IL-4 and IFN-\( \gamma \) induced MCH class II expression on monocytes.19 Since it is produced by Th2 cells and inhibits Th1 function by preventing T cells cytokine production, IL-10 is regarded as a T cell cross-regulator factor, and has, thus, been referred to as an ‘anticytokine’.20 In *in vitro* Pb is actually able to induce activation of Th2 lymphocytes,21 so providing a possible explanation of plasma IL-10 increase in low Pb-exposed workers in the study. In very low Pb-exposed workers, ie, pottery workers, plasma IL-10 levels was not significantly different with respect to non-exposed workers. The significant positive correlation between Pb-B and IL-10 level seems to suggest a dose-dependent increase by lead. Finally, Pb can determine an increase of plasma IL-10, even after correction for main confounding factors, confirming possible interference of Pb in the complex pathogenesis of immunophlogosis through mechanisms not yet clearly understood.

The mean TNF-\( \alpha \) levels were significantly different between the three groups of workers, but only low Pb-exposed workers (Pb-B = 9.1–46.0 \( \mu \)g/dL) had a tendency to higher plasma levels than non-exposed...
workers (Pb-B = 1.0–11.0 μg/dL). The lack of significant difference in TNF-α levels between very low Pb-exposed workers (Pb-B = 3.2–18.0 μg/dL) and non-exposed workers can be due to the wide dispersion of measures, but also to the overlapping of Pb-B levels of the two groups (Tables 2 and 3). Due to the small sample size of pottery group, no further statistical analysis was performed. Thus, Pb seems to favour the production of TNF-α at low, but not at very low blood levels. This effect is also confirmed when considering the main confounding factors. In experimental studies, Pb has been shown to cause a significant increase of TNF-α production, both in vitro by macrophages,16 and rat spleen cells,10 and in vivo in rats,22,23 and cows.24 In particular, Pb induces an increase of TNF-α at low doses and a decrease at higher doses, as demonstrated in human peripheral blood mononuclear cells,25 in rats,23,26 and in exposed workers compared to a non-exposed controls.27 It should be noted that the Pb-B levels of exposed workers examined by Yucesoy et al.,27 (92–94 μg/dL) were almost all above those measured in the foundry workers of our survey. TNF-α is a cytokine mainly involved in chronic cell-mediated inflammation.17 It is produced by activated macrophages/monocytes, fibroblasts, mast cells, and some T and natural killer cells.28,29 Pb has been found to enhance in vitro production of TNF-α by human peripheral mononuclear cells.30 The stimulatory effect of Pb appears to be due to a modification of the expression of membrane TNF-α receptors, which are modulated by Pb. In fact, it is known that Pb has a high affinity for cysteine-rich membrane proteins, and it may interact with membrane receptors. In previous experiments in vitro, we have shown that Pb modifies membrane fluidity of erythrocytes of Pb-exposed workers, as well as membrane fluidity of neutrophils.2,31 Moreover, TNF-α is able to increase the production of IL10. In our Pb-exposed workers, the increased concentration of IL-10 might be secondary to the increased production of TNF-α. In fact, in all 116 workers considered together, a significant correlation was found between the levels of these two cytokines (rho = 0.204; P < 0.05).

No changes in other cytokines levels were found in the workers examined, probably due to the low Pb-B levels of exposed workers. Previous reports showing, for example, decreased IFN-γ production, had been carried out in Pb-exposed workers with higher Pb-B levels than the pottery or foundry workers of our study.27–32

No differences of NO plasma concentration was found between the three groups of workers examined. There is no agreement about this subject between experimental studies. According to Vaziri et al.,33 Pb exposure decreases NO availability, whereas other authors found that low concentrations of Pb is able to stimulate in vitro NO production,10,11,34 and that the stimulatory effect reduces itself with increasing Pb concentration. A study conducted on healthy males and females with environmental Pb exposure (Pb-B levels always <10 μg/dL) found no significant correlation between Pb-B and plasma NOx levels.35 Therefore, it seems that Pb-B levels, currently measured in a group of subjects with moderate environmental Pb-exposure and in the two groups of Italian occupationally-exposed workers examined in this study, do not induce significant changes in NOx production by macrophages, endothelial cells and platelets.

In conclusion, we found that at low Pb concentrations, cytokine balance is modified with an increase of plasma TNF-α and IL-10 levels. These two cytokines have different biological effects which are almost, in part, contrasting and interconnected. It could be hypothesised that the system of immunophlogosis of exposed workers in response to low Pb-B concentrations shows an increase of pro-inflammatory cytokines, such as TNF-α, with a consequent increase of other cytokines, such as IL-10, considered a T cell cross-regulatory factor. Further studies on larger groups of exposed workers with different levels of Pb exposure are needed to confirm these results, in order to explain how this metal acts on different components of the inflammation system, and on their complex homeostasis mechanisms.

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