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Comparison of lead and copper exposure effect on immune cells in mice

Arvydas Markevièius*, Aldona Dringelienë

Institute of Immunology of Vilnius University, Molëtø pl. 29, LT-08409 Vilnius 21, Lithuania

Background. Effects of environmental exposure on the immune system may appear a major health problem. The aim of our studies included a comparative assessment of the effects of lead and copper exposure on immune markers of lymphocytes in mice as an experimental model.

Methods. The effect of prolonged (19 weeks) dietary lead and copper excess was studied in mouse (BALB/c). The control group was supplied with pure drinking water and two experimental groups with solutions: 3 mg/L $Pb(NO_3)_2$ and 1180 mg/L $CuSO_4 \cdot 5H_2O$. Blood samples were analysed flow-cytometrically for lymphocyte markers ($CD3^+$, $CD19^+$, $NK\text{-}1.1^+$, $CD4^+CD8^-$, $CD8^+CD4^-$, $CD4^+CD8^+$).

Results. An average lead and copper intake calculated on the basis of liquid quantities consumption was 0.23 mg Pb/kg and 42 mg Cu/kg (body weight)/day, respectively. Multiparameter flow cytometry analysis demonstrated that lead consumption reduced the amount of $CD4^+CD8^-$ (helper) cells (1.3-fold, $P < 0.05$) and increased the content of $CD8^+CD4^-$ (suppressor) cells (1.4-fold, $P < 0.005$). Copper consumption did not influence the $CD4^+CD8^-$ subpopulation, however, it suppressed the $CD8^+CD4^-$ cell subpopulation and increased the immunoregulatory index from 1.44 to 1.85. Lead did not influence the natural killer cell population, but copper excess reduced this population 1.3-fold.

Conclusions. The content of double-positive lymphocytes $CD4^+CD8^+$ (progenitors of T cells) was increased in lead-treated mice but suppressed by copper excess. Our results have revealed that lead and copper excess affects *in vivo* the subpopulations of lymphocytes of mice in different manner.

Key words: lead, copper, exposure, lymphocyte subpopulation, flow cytometry, mice

INTRODUCTION

Environmental pollutants are believed to be factors adversely affecting animal and human organisms. One mechanism whereby various hazardous chemicals (airborne and solid particles) alter the functioning of exposed individuals is the modulation of biochemical and immune homeostasis. The immune system consists of a complex network of cells and molecules scattered throughout the body of all multicellular organisms and able to recognize and neutralize potentially harmful agents, conferring to the organism resistance to infectious and malignant diseases. Optimal functioning of the immune system requires the intersection among specific cells and cell products in a sequential highly regulated manner. Effects of environmental exposure on the immune system may appear a major health problem. The presence of environmental contaminants in air, water and food may cause significant health risks to the exposed human population. However, problems associated with assessing of chronic exposure to low doses of environmental chemicals, multiple routes of exposure, diseases with long latency periods and nonspecific health outcome impede the appropriate human epidemiological studies. Therefore, it would be useful to complement human epidemiology with experimental animal studies.

Determination of the number of various lymphocyte types and concentration of immunoglobulin classes in the peripheral circulation gives relevant information on the cellular and humoral immune responses of the studied individual or population. Various environmental factors can invoke the bias in the immune response of T lymphocytes, natural killer (NK) and antigen presenting cells as well as of humoral immunity chains. Investigating alterations in the immunity can, therefore, yield relevant information on the relationship between exposure to environmental

*Corresponding author. E-mail: arvmark@imi.lt

contaminants and susceptibility to various infectious diseases (1–3).

Chronic exposure to low levels of lead has been a matter of public health concern in many countries (4). Although Pb poisoning has been studied for years, some of the toxic effects still cannot be explained (5).

Copper is known to play an important role in the development and maintenance of the immune system, but the exact mechanism of its action is not yet known (6). Copper insufficiency is often associated with severe pathological alterations including impairment of blood, liver and immune system parameters (7). Inaccurate or intentional excessive copper ingesting may lead to toxic effects in human (8). At high doses, this element can be acutely lethal; at lower doses its effects include mutagenicity, carcinogenicity, teratogenicity, immunosuppression, poor body condition, and impaired reproduction (9). Animals monitored or evaluated *in situ* for the appropriate suite of endpoints can provide information about both exposure levels and potential adverse effects (10). Evidently important would be to assess whether there is any relationship between exposure of these pollutants and immune system biomarkers in animals.

The aim of our studies included a comparative assessment of the effects of lead and copper exposure on immune markers of lymphocytes in mice.

MATERIALS AND METHODS

Animals and maintenance. Male BALB/c mice (10 weeks old) were obtained from the vivarium of the Institute of Immunology (Vilnius, Lithuania). After acclimatization (2 weeks) the animals were housed in solid-bottomed cages containing bedding of wood shavings and were allowed food and water *ad libitum*. The room temperature was maintained at 21–24 °C and a 12/12 h light/dark cycle was employed. The mice were divided at random into three equal groups of 10 animals each. During the experimental period of 19 weeks, the control group (group A) was supplied with pure drinking water and 2 experimental groups with solutions: group B – 3 mg/l $Pb(NO_3)_2$, *i.e.* 1.8 mg Pb/l, group C – 1180 mg/l $CuSO_4 \cdot 5H_2O$, *i.e.* 300 mg Cu/l (metal salts were an. gr. from Fluka, Germany) Lead and copper consumption was recorded twice a week. At the end of the experiment, the mice were sacrificed by cervical dislocation and their organs were weighed. Approval of the Lithuanian Ethic Committee for Laboratory Animal Use was obtained prior to commencement of the experiments.

Lead and copper analysis. Blood samples with heparin as an anticoagulant and livers homogenized without any additives were analysed by atomic absorption spectrometry using a Shimadzu AA-6800 (Japan) instrument.

Flow cytometry analysis. White blood cells were isolated by the routine method using ammonium chloride lysing solution and processed for flow cytometry testing according to the standard direct labeling method (11). Cells suspended to a concentration of 10^6/ml were stained in the dark for 30 min with 1 μg of each appropriate labelled monoclonal antibodies: CyChrome-antiCD3e, FITC-antiCD4, PE-antiCD8a, FITC-antiNK (NK-1.1$^+$), PE-antiCD19 (BD Pharmingen, Germany). Isotypical control was performed with an appropriate labeled irrelevant immunoglobulins and Fc Block was used as required. Multiparameter flow cytometric analysis was performed on a

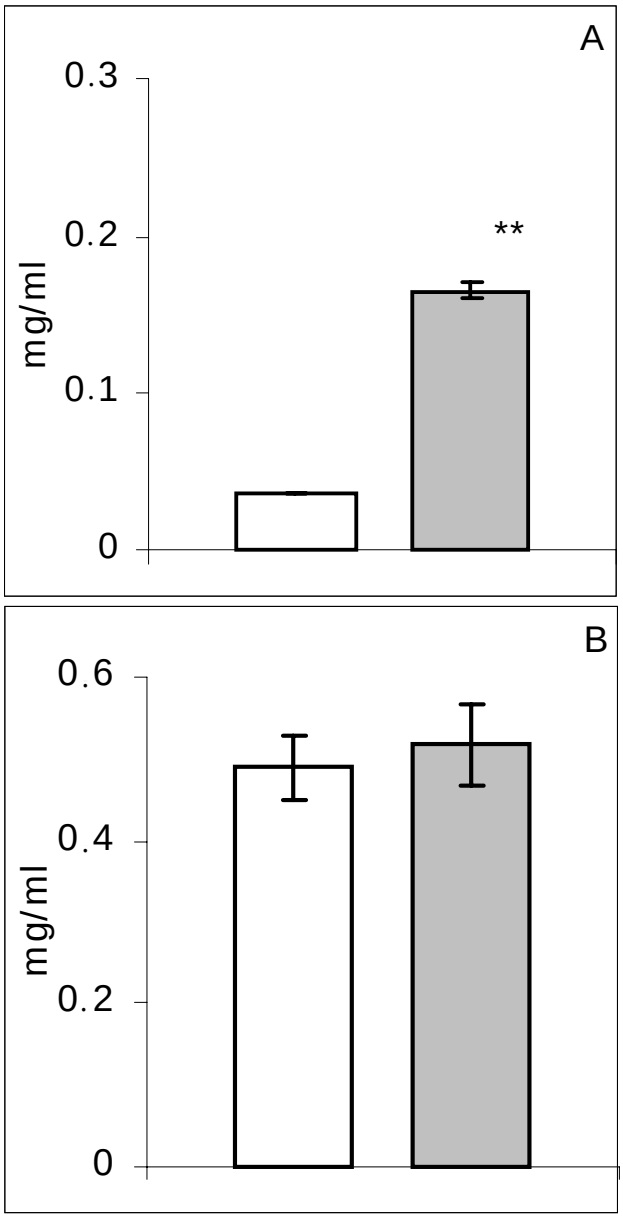


Figure. Lead (A, C) and copper (B, D) concentration in the blood (A, B) and liver (C, D) of mice. □ – control group, ▒ – experimental group. * P < 0.05. ** P < 0.005 compared with control mice.

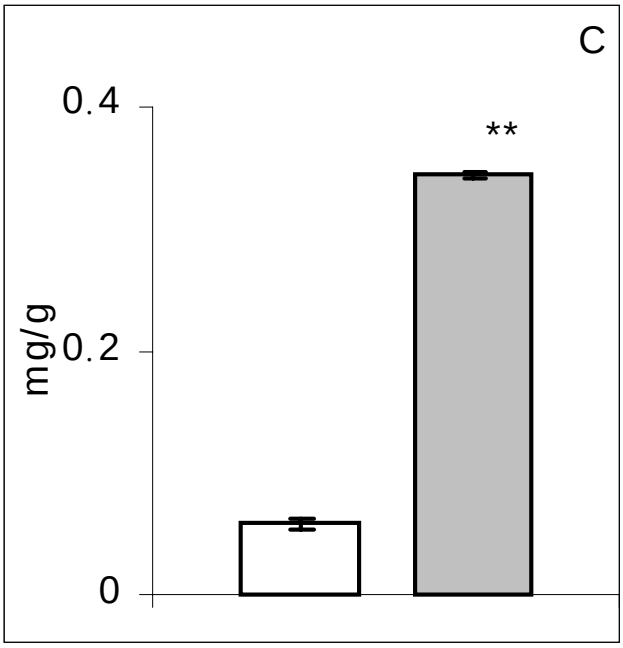
FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems, San José, California, USA). Data were acquired using CELLQuest software. $CD3^+$, $CD19^+$, NK-1.1^+ populations and $CD4^+CD8^-$, $CD8^+CD4^-$, $CD4^+CD8^+$ subpopulations of lymphocytes were investigated.

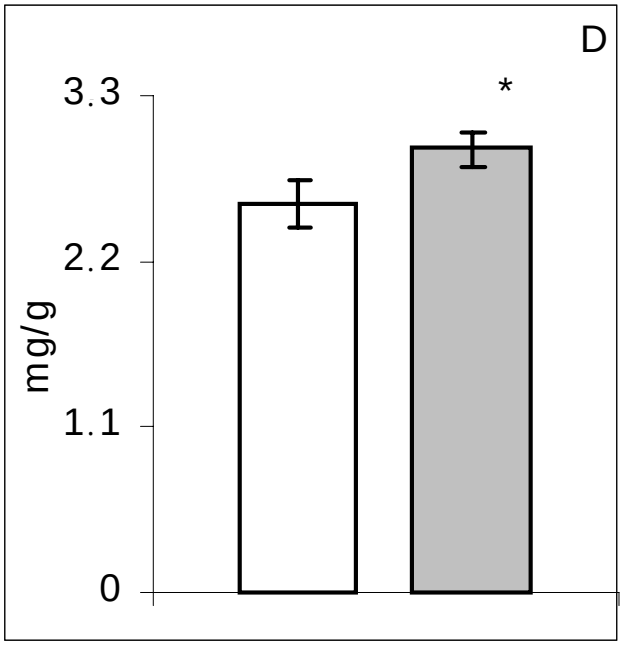
Data analysis. Statistical analysis was performed using the Microsoft Excel Ver. 7.0 computer program. The data were expressed as mean values (M) ± standard deviation (SD). Statistical differences among the groups were assessed by the Student's t test. The results were considered significant at $P < 0.05$.

Table. **Percentage distribution of lymphocyte populations from total gated lymphocytes in the blood of mice according to immunomarkers**

Immunomarkers	Group A	Group B	Group C
$CD3^+$	46.87 ± 3.56	36.63 ± 3.84**	44.38 ± 5.66
$CD4^+$ $CD8^-$	27.81 ± 3.53	21.55 ± 3.98*	27.76 ± 4.71
$CD4^+$ $CD8^+$	0.21 ± 0.027	0.418 ± 0.15*	0.08 ± 0.02**
$CD8^+$ $CD4^-$	19.29 ± 1.64	26.82 ± 4.00**	14.98 ± 2.21**
$CD19^+$	34.99 ± 2.67	32.73 ± 4.37	33.57 ± 5.14
NK-1.1^+	14.28 ± 2.15	14.49 ± 2.01	11.36 ± 1.56*
$CD4^+CD8^-/CD8^+CD4^-$	1.44 ± 0.20	0.81 ± 0.21*	1.85 ± 0.21*

Groups: A – control, B – 1.8 mg Pb/l, C – 300 mg Cu/l.
* $P < 0.05$. ** $P < 0.005$ compared with control mice.





RESULTS

An average lead and copper intake calculated on the basis of liquid consumption was 0.23 mg Pb/kg body weight/day (in group B) and 42 mg Cu/kg bw/day (in group C). The exposure to Pb^{2+} excess resulted in an increase of the concentration of this metal in the blood and its accumulation in the liver: the blood lead level in experimental group was 4.5 times and the liver level 7 times higher than in control group (Figure). There were no significant differences in blood copper concentrations between control and experimental mice groups.

We investigated $CD3^+$, $CD19^+$, natural killer (NK-1.1^+) populations and $CD4^+CD8$-, $CD8^+CD4$-, $CD4^+CD8^+$ subpopulations of lymphocytes and quantified their relative proportions using double parameter analysis by flow cytometry (Table). The main population of lymphocytes in the blood of all mice groups comprised T cells with $CD3^+$marker. $CD3^+$ cells were significantly suppressed in Pb^{2+}-treated mice ($P < 0.005$). Our investigation showed that $CD3^+$ population was not influenced by copper excess.

Cellular immune system subpopulation analysis showed that in Pb^{2+}-treated mice $CD4^+CD8$- (helper) cells were suppressed (1.3-fold, $P < 0.05$) while $CD8^+CD4$- (suppressor) cells were upregulated (1.4-fold, $P < 0.005$). The $CD4^+CD8$- subpopulation was not influenced by copper excess, but $CD8^+CD4$- cells were suppressed ($P < 0.005$). This process resulted in a decrease of the immunoregulatory index (the ratio of $CD4^+CD8$-/$CD8^+CD4$-) from 1.44 in control mice to 0.81 in Pb^{2+}-treated mice and an increase to 1.85 in copper excess treated mice ($P < 0.05$). $CD4^+CD8^+$ lymphocytes were detected in blood of all mice in very small quantities, and administration of Pb^{2+} significantly increased (2.0-fold) this population, while administration of copper significantly suppressed it (2.6-fold).

The population of $CD19^+$ (B lymphocytes) was not altered by Pb^{2+} or Cu^{2+} excess.

Natural killers (NK-1.1^+) were not altered by Pb^{2+} excess, but Cu reduced the NK-1.1^+ values. NK-1.1^+ cells in blood were found 1.3 times below the average in group C mice in comparison with the control group.

DISCUSSION

The concentration of Pb in the blood and liver of mice exposed for a prolonged period was elevated and this fact showed accumulation of lead in the organism. Surprisingly, copper did not show a substantial accumulation in the blood and liver of mice. These differences imply a different mechanism of metabolism and retention of lead and copper ions in murine organism.

Using multiparameter analysis by flow cytometry, we were able to identify three populations and three subpopulations of lymphocytes as well as to quantify their relative proportions (Table). The main population of lymphocytes in the blood of all groups of mice comprised T cells with $CD3^+$ marker. We observed a reduction of T-lymphocyte subpopulation ($P < 0.05$) in group B of mice that had received an excess of lead ions, and this result was in agreement with the publication of Pinkerton et al. (12).

A recent study (13) designed to answer the question on immunological effects to Pb^{2+} exposure showed a positive correlation of Pb^{2+} concentration in blood with IgG level but no effect on T cell population. Several other occupational studies have observed controversial associations between higher blood lead levels and some immune markers: lower levels of T cells and T-helper cells and higher levels of T-suppressor cells (12), lower levels of IgG, IgM, complement, and T-helper cells, but no change in T-suppressor, B, and NK cells (14).

We can also prove a lower $CD4^+$ and a higher $CD8^+$ cell proportions in the blood of mice after a long-term exposure to low doses of Pb inorganic salt. Altered ratios of Th1 and Th2 cells are also observed in immunodisregulations, leading to impaired cell-mediated immunity with an increased incidence of infectious diseases or cancer and/or aberrant immunity that could culminate in an autoimmune disease. Lead induced an oligoclonal T cell response in mice, which could be initiated by self-antigens and was predominantly type 2; it may be responsible for autoantibody production and the detrimental health effects associated with Pb exposure (15).

Pb enhances specific T cell proliferation through an indirect mechanism, and a single exposure to Pb during alloantigen priming elicited a population of $CD4^+$ cells (16).

Copper excess administration changed the proportions of helper ($CD4^+CD8^-$) and suppressor ($CD8^+CD4^-$) cells and increased the immunoregulatory index. This phenomenon may be considered to be copper concentration dependent as observed by us in experiments with mice exposed to different Cu concentrations in drinking water (17).

The amount of double positive lymphocytes $CD4^+CD8^+$ (progenitors of T cells) was increased in Pb^{2+}-treated mice but suppressed by copper excess. It is possible that low doses of lead stimulate proliferation of these cells not only *in vitro* (18, 19) but also *in vivo*. A significant excess of copper adversely influenced the proliferation and differentiation processes of lymphocyte subpopulations.

A low-dose prolonged treatment with lead did not influence the number of NK-1.1^+ cells in the blood of mice. Failure of inorganic lead in a low-level short exposure to impair natural killer (NK) cell functions in rats had been determined previously (20), but a prolonged exposure (10 weeks) revealed a suppressed NK cell cytotoxicity (21).

The excess of Cu reduced the NK-1.1^+ values and the main subpopulation of the innate immunity NK cells was affected substantially (a 1.3-fold decrement). It is demonstrated that NK T cells, like other effective natural cytotoxic cells of the organism, are derived from $CD4^+CD8^+$ thymocytes which escape apoptosis in thymus (22) and migrate into blood stream (23). Therefore, we conclude that excessive and prolonged intakes of copper significantly suppress the proliferation of cytotoxic NK cells and the progenitors of NK T cells ($CD4^+CD8^+$) in mice.

In summary, observations made in this study, together with previous findings (14), indicate that lead-induced effects might cause impairment in $CD4^+$ cell proliferation and act immunosuppressively by changing the proportions of lymphocyte subpopulations.

The immune system is a relatively sensitive system, and a prolonged lead and copper intake initiates disturbances in immune homeostasis. The ensuing consequences of such disturbances are the transformed proportions of lymphocyte subpopulations and phenotypic properties of immunocompetent cells.

On the basis of our and other results, we can conclude that lead and copper affect *in vivo* the subpopulations of immune system cells in different manner.

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ÐVINO IR VARIO POVEIKIO PELIØ IMUNINËS SISTEMOS LÀSTELËMS PALYGINIMAS

Santrauka

Ðiame darbe, kuris yra kompleksinio tyrimo dalis, nagrinëjome ilgalaiká (19 savaièiø) ðvino ir vario poveiká imunokompetentiniø làsteliø fenotipinëms savybëms ($CD3^+$, $CD19^+$, $NK\text{-}1.1^+$, $CD4^+CD8^-$, $CD8^+CD4^-$, $CD4^+CD8^+$) *in vivo* peliø (BALB/c) modelinëje sistemoje. Vidutinis ðvino ir vario suvartojimas, apskaièiavus iðgerto tirpalo, kieká, sudarë 0,23 mg Pb ir 42 mg Cu 1 kg kûno svorio per parà. Daugiaparametrinës tëkmës citometrijos tyrimø duomenimis, dël ðvino poveikio sumaþëjo $CD4^+CD8^-$ (helperiniø) làsteliø (1,3 karto, $P < 0,05$) ir padaugëjo $CD8^+CD4^-$ (supresoriniø) làsteliø (1,4 karto, $P < 0,005$). Varis nepaveikë $CD4^+CD8^-$ làsteliø subpopuliacijos, bet slopino $CD8^+CD4^-$ làsteliø subpopuliacijà ir padidino imunoreguliacijos indeksà nuo 1,44 iki 1,85. Ðvinas neturëjo átakos natûraliø killeriniø làsteliø populiacijos kiekiui, bet vario perteklius ðià populiacijà sumaþëjo 1,3 karto. Dvigubai þymëtø $CD4^+CD8^+$ limfocitø (T làsteliø pirmtakø) kiekis padidëjo dël ðvino poveikio, tuo tarpu vario perteklius sumaþino ðià populiacijà. Iðvada – ðvino ir vario perteklius *in vivo* skirtingai paveikia imuninës sistemos subpopuliacijas.

Raktaþodþiai: ðvinas, varis, poveikis, limfocitø subpopuliacijos, tëkmës citometrija, pelës