Cytokine Production by Human Peripheral Blood Mononuclear Cells after Exposure to Heavy Metals

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The effects of heavy metal exposure on the immune system were determined by measuring cytokine production of human peripheral blood mononuclear cells (*h*-PBMCs) from a healthy female. The *h*-PBMCs were exposed for 3 d to $CdSO_4$, $K_2Cr_2O_7$, and $HgCl_2$ at 1, 5, 10 μM and to $(CH_3COO)Pb$ at 10, 50, and 100 μM. Concentrations of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β, IL-6, IL-8, and IL-10 were quantified using the ELISA method. The results showed that the cytokines assayed were differentially affected by heavy metal exposure. Chromium significantly increased the production of IL-1β while decreasing the production of IFN-γ, IL-6, IL-8, and IL-10. Mercury at low concentrations increased the levels of TNF-α and IL-1β. At higher concentrations, an opposite effect was seen. Cytokines may function as biomarkers in elucidating the mechanisms of the immunotoxic effects of heavy metals.

Key words —— cytokine, heavy metal, human peripheral blood mononuclear cell

INTRODUCTION

Heavy metals are ubiquitously present in the environment. Human exposure may result from sources in the industrial setting. Sources of nonoccupational exposure are from contaminated air, food, water, and soil. Immunotoxicity is among the many health effects of heavy metal exposure. The most immunosuppressive of the heavy metals was found to be mercury based on the results of animal experiments. Copper, manganese, cobalt, cadmium, and chromium, in decreasing potential for immunosuppression, share the same properties.[1)]

The immune system effects of heavy metals are varied. Autoimmune glomerulonephritis was demonstrated with chronic exposure of rats to $HgCl_2$.[2)] Inflammatory cell infiltration may play an important role in producing renal damage from cadmium exposure.[3)] The resistance of the kidneys to the cytotoxicity of cadmium has been proposed to involve metallothioneins, the levels of which may be affected in a dose-dependent manner by IL-1β.[4,5)] Hexavalent chromium was found to inhibit B and T cell responses in the splenocytes of exposed rats.[6)] Contact dermatitis and cancer are other effects of chromium that may involve the immune system.[7)]

The effects of lead on the immune system are not well documented. Nonetheless, reports of reduced resistance and increased mortality in experimental animals have been published following infection with bacterial and viral agents.[8)] Lead exposure resulted in an increase in the number of plaque-forming cells as a response to sheep red blood cells, which may be taken as an indicator of immunotoxicity.[1)]

Despite the numerous reports on the effects of heavy metals on the immune system, the mechanism of the changes in the immune response remains unclear. Cytokines are important immune response modulators that may be affected by heavy metal exposure. The present study attempts to provide additional evidence on the immune system effects of heavy metals. The cytokine profile of human peripheral blood mononuclear cells (*h*-PBMCs) following exposure to cadmium, chromium, inorganic mercury, and inorganic lead was determined.

MATERIALS AND METHODS

Venous blood was extracted from the cubital vein of a healthy female volunteer after informed consent was obtained. *h*-PBMCs were separated from the venous blood by the method described by Jonai

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et al.[9] The concentration of *h*-PBMCs was adjusted to 1.5×10^6 cells/ml with RPMI 1640 containing 10% fetal calf serum (FCS), 100 μg/ml of streptomycin and 100 units/ml of penicillin. The cell suspension was divided into three 4-cm^2 wells for each concentration of the heavy metal and the corresponding three 4-cm^2 wells for the control.

$CdSO_4$, $K_2Cr_2O_7$, $HgCl_2$, and $(CH_3COO)Pb$ were used for cadmium, chromium, inorganic mercury, and inorganic lead exposure, respectively. Concentrations used for cadmium, chromium, and mercury exposure were 1, 5, and 10 μM. Ten, 50, and 100 μM concentrations were used for the lead exposure. All heavy metals were reagent grade and purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. All cell groups were cultured at 37°C in 5% CO_2 for 3 d. On the third day, the cell suspension from each well was centrifuged and the supernatant was collected for cytokine assay.

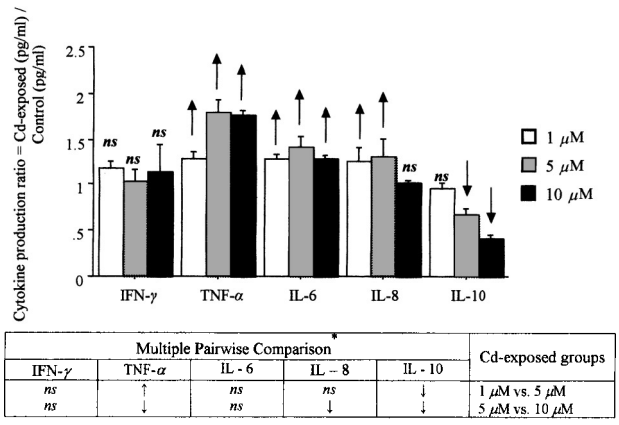
The levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, and IL-10 in the supernatant were measured by the ELISA method using cytokine kits (CytoscreenUS™ from Bio Source, Camarillo, California, U.S.A.). Absorbance was measured with a microplate reader (Iwaki SME400, Funabashi, Japan).

The differences in the levels of the cytokin between the control group and the heavy metal-exposed groups were estimated by repeated measures analysis of variance (ANOVA) in conjunction with multiple pairwise comparisons. Statistical significance was set at $p < 0.05$.

Consistency in cytokine production from metal exposure was verified by a second experiment using *h*-PBMCs from the same individual. Experiment 2 followed the protocol described above. Trends in cytokine levels in both experiments were examined and compared. Only consistent results were subjected to statistical analysis and are shown in the figures below.

RESULTS

Figures 1–4 show the levels of the cytokines assayed in the heavy metal-exposed groups relative to the control group. Cadmium exposure slightly induced production of TNF-α and IL-6 at all concentrations (Fig. 1). The increase in IL-8 levels was noted up to the 5 μM concentration. Suppression of IL-10 levels was noted for the 5 and 10 μM concentrations. No significant differences in the IFN-γ levels were noted between control and Cadmium-exposed *h*-PBMCs at any concentration.



Multiple Pairwise Comparison*					Cd-exposed groups
IFN-γ	TNF-α	IL - 6	IL – 8	IL - 10	
ns	↑	*ns*	*ns*	↓	1 μM vs. 5 μM
ns	↓	*ns*	↓	↓	5 μM vs. 10 μM

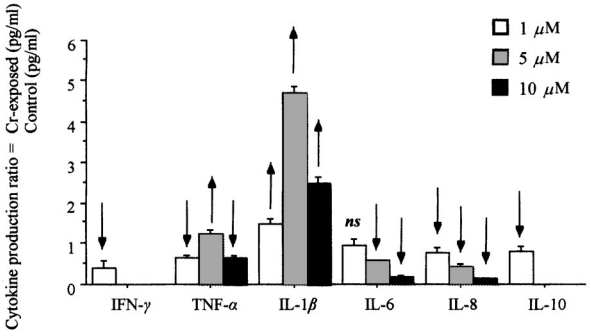
Fig. 1. Ratio of Cytokine Production in Cadmium-Exposed *h*-PBMCs to That in Control *h*-PBMCs

h-PBMCs exposed to cadmium at concentrations of 1, 5, and 10 μM). ***ns***: no statistically significant difference in the cytokine production of cadmium-exposed and control cells. ↑: cytokine production in exposed cells > control cells; $p < 0.05$. ↓: cytokine production in exposed cells < control cells; $p < 0.05$. *: Multiple comparison Tukey's test. ***ns***: no statistically significant difference between cytokine production of two cadmium-exposed groups. ↑: cytokine production greater in the group exposed to higher concentration of cadmium; $p < 0.05$. ↓: cytokine production less in the group exposed to higher concentration of cadmium; $p < 0.05$.

Figure 2 shows the relation between cytokine levels and chromium exposure. A decrease in the levels of most cytokines assayed in the exposed group compared to the control was noted. IL-1β and TNF-α were exceptions. Chromium exposure enhanced IL-1β production. More than a 5-fold increase in IL-1β level was noted at the concentration of 5 μM. Production of TNF-α was slightly stimulated at 5 μM, but inhibited at 1 and 10 μM.

Mercury exposure significantly increased the production of TNF-α, IL-1β, IL-6, and IL-8 at 1 and/or 5 μM concentrations (Fig. 3). Inhibition was noted at 10 μM for all the cytokines assayed, although it barely reached significance for TNF-α. Inhibition of IL-10 production was also noted at 5 and 10 μM concentrations.

The response of *h*-PBMCs to inorganic lead exposure was consistent for TNF-α and IL-6. The production of these cytokines was enhanced by inorganic lead exposure at concentrations greater than 10 μM (Fig. 4).

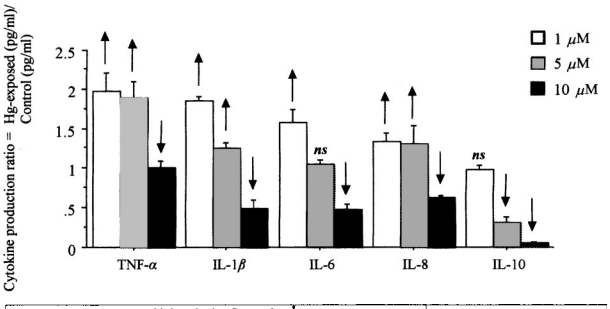


Multiple Pairwise Comparison*						Cr-exposed groups
IFN - γ	TNF - α	IL - 1β	IL - 6	IL - 8	IL - 10	
-	↑	↑	↓	↓	-	1 μM vs. 5 μM
-	↓	↓	↓	↓	-	5 μM vs. 10 μM

Fig. 2. Ratio of Cytokine Production in Chromium-Exposed *h*-PBMCs to That in Control *h*-PBMCs

h-PBMCs exposed to chromium at concentrations of 1, 5, and 10 μM. ***ns***: no statistically significant difference in the cytokine production of chromium-exposed and control cells. ↑: cytokine production in exposed cells > control cells; $p < 0.05$. ↓: cytokine production in exposed cells < control cells; $p < 0.05$. *: Multiple comparison Tukey's test.

ns: no statistically significant difference between cytokine production of two chromium-exposed groups. ↑: cytokine production greater in the group exposed to higher concentration of chromium; $p < 0.05$. ↓: cytokine production less in the group exposed to higher concentration of chromium; $p < 0.05$.



Multiple Pairwise Comparison*					Hg-exposed groups
TNF - α	IL - 1β	IL - 6	IL - 8	IL - 10	
↓	↓	↓	↓	↓	1 μM vs. 5 μM
↓	↓	↓	↓	↓	5 μM vs. 10 μM

Fig. 3. Ratio of Cytokine Production in Inorganic Mercury-Exposed *h*-PBMCs to That in the Control *h*-PBMCs

h-PBMCs exposed to inorganic mercury at concentrations of 1, 5 and 10 μM. ***ns***: no statistically significant difference in the cytokine production of inorganic mercury-exposed and control cells. ↑: cytokine production in exposed cells > control cells; $p < 0.05$. ↓: cytokine production in exposed cells < control cells; $p < 0.05$. *: Multiple comparison Tukey's test.

ns: no statistically significant difference between cytokine production of 2 mercury-exposed groups. ↑: cytokine production greater in the group exposed to higher concentration of mercury; $p < 0.05$. ↓: cytokine production lesser in the group exposed to higher concentration of mercury; $p < 0.05$.

DISCUSSION

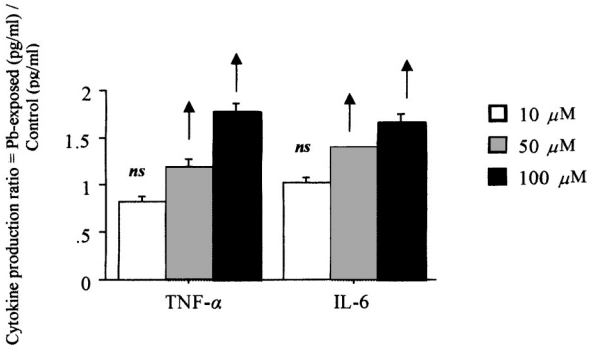
Multiple pairwise comparisons were performed to determine the direction of change in cytokine levels with exposure to different concentrations of heavy metals. Dose–response relationships were noted for some cytokines as concentrations of the heavy metals increased. A decreasing trend was noted in IL-10 production with increasing concentrations of cadmium (Fig. 1). A strong inhibitory effect was exhibited by chromium, especially at concentrations above 1 μM. IFN-γ and IL-10 were undetectable at these concentrations (Fig. 2). A definite negative relationship was noted between chromium and IL-6. The same relationship was seen between chromium and IL-8.

A negative relationship between inorganic mercury concentrations and cytokine levels was also seen for TNF-α, IL-1β, IL-6, IL-8, and IL-10 (Fig. 3). The relationship was the opposite for inorganic lead (Fig. 4). Higher levels of TNF-α and IL-6 were noted with increasing inorganic lead concentrations.

The acute phase of the immune response involves the cytokines assayed in the present study. TNF-α and IL-1β produce inflammatory cell infiltration, IL-8 is involved in chemotaxis, antibody production is induced by IL-10, both IFN-γ and IL-6 have cytotoxic effects.[10] Detection of changes in the levels of cytokines could potentially be used as biomarkers or early indicators of tissue injury or alterations in the immune response.

Earlier studies suggested that the heavy metals investgated in the present study can alter certain immune system parameters. However, the mechanisms and specific immune system components affected by the heavy metals were not clearly defined. *In vitro* studies revealed that cadmium causes immunomodulation by suppressing natural killer cell activities and the cytotoxicity of peripheral blood lymphocytes, thereby affecting the ability to combat infection.[11,12] Furthermore, intense inflammatory cell infiltration has been noted in cases of severe kidney damage from cadmium exposure. Mercury has been shown to suppress human T lymphocyte proliferation, affecting susceptibility to metal toxicity.[13]

The relationship between immune system response and heavy metal exposure is also not so clear. T-helper 1 cell activation was inhibited while T-helper 2 cell activation was enhanced by lead.[1] Nonetheless, the possibility of immune response suppression that will render the organism more susceptible to infections and cancer exists. Because of the complex nature of the immune system with its multiple components of redundant or opposing effects, the interpretation of data from studies involv-



Multiple pairwise comparison*		Pb-exposed groups
TNF - α	IL - 6	
↑	↑	10 μM vs. 50 μM
↑	↑	50 μM vs. 100 μM

Fig. 4. Ratio of Cytokine Production in the Inorganic Lead-Exposed *h*-PBMCs to the Control *h*-PBMCs

h-PBMCs exposed to inorganic lead at concentrations of 10, 50, and 100 μM. ***ns***: no statistically significant difference in the cytokine production of inorganic lead-exposed and control cells. ↑: cytokine production in exposed cells > control cells; $p < 0.05$. ↓: cytokine production in exposed cells < control cells; $p < 0.05$. *: Multiple comparison Tukey's test.

ns: no statistically significant difference between cytokine production of two lead-exposed groups. ↑: cytokine production greater in the group exposed to higher concentration of lead; $p < 0.05$. ↓: cytokine production less in the group exposed to higher concentration of lead; $p < 0.05$.

ing limited immunologic assays is likewise complicated. The considerable interindividual differences in immune response may also affect the varied effects noted among the studies conducted in this area.

The present study used four heavy metals at concentrations that did not produce overt cellular death. The concentrations used were below the concentrations used by Yamada *et al.*[14] in an experiment that determined direct cytotoxicity of various metals, including cadmium, mercury, and lead, by the uptake of [^{35}S]Cys. No inhibition in the uptake was noted for lead up to the concentration of 200 μM. It was also shown that mercury uptake of [^{35}S]Cys was inhibited only from 20 μM. Jonai *et al.*[15] estimated cellular viability by adding trypan blue solution to suspensions of control and cadmium-exposed human lymphocytes. They found no significant differences between the number of viable cells in the control and exposed group up to 20 μM. Furthermore, this experiment showed that heavy metals affected cytokine production at both low and high concentrations.

Another supposition proposed by the present study is that heavy metals may produce deviations from the normal immune system reaction at the cellular level. Significant differences were noted in the cytokine production of control cells and heavy metal-exposed cells. Kondo *et al.*[5] related the presence of IL-1β levels to increased resistance to the cytotoxic effects of cadmium possibly by a mechanism involving metallothioneins. Although our experimental findings did not show an increase in IL-1β secondary to cadmium exposure, an elevated IL-1β level was noted for *h*-PBMCs exposed to mercury, another metal known to induce metallothionein production. Also in the present study, the promotion and inhibition of cytokine production at low and at high concentrations of heavy metals, respectively, may represent a protective reaction of cells against injurious agents. This reaction was remarkably demonstrated by chromium. Inorganic lead-exposed cells also manifested this phenomenon, although at higher concentrations.

The inhibition of B and T lymphocytic responses has been ascribed to chromium exposure.[6] In the present study, all the cytokines assayed, except for IL-1β, suggested an inhibitory effect of chromium at all concentrations used. IFN-γ, TNF-α, IL-6, and IL-10 are produced by either or both B and T lymphocytes. IL-8 is produced mostly by mast cells. In the case of IL-1β, macrophages and neutrophils are mostly responsible for its production.[10] This may explain the largely disparate results in the levels of IL-1β and the other cytokines assayed. Evidence of the inhibition of B and T cell function was likewise shown by Wang *et al.*[16] when they attempted to determine the role of chromium in the increased risk of infection in patients using joint prostheses. This alteration in immune response was ascribed to the inhibition of IFN-γ and IL-6 production.

Our results showed that exposure of *h*-PBMCs to mercury produces an increase in the levels of all the cytokines assayed except for IL-10, for which a decrease was noted. At concentrations below 4 μM, Warbrick *et al.*[17] did not note any inhibition of the expression of the IL-8 and TNF-α mRNA for in human leukemic mast cells incubated with $HgCl_2$. The results of our present study indicate that inhibition may take place at concentrations higher than previously shown, since suppression of the two cytokines was noted at concentrations above 5 μM. Moreover, the disagreement in the results of two experiments may result from the differences in the predominant cell population studied. Warbrick and coworkeres' experiment utilized only mast cell, while the present study used mononuclear cells without further cell separation.

Little is known about the effect of inorganic lead on the immune system. A review by Bernier *et al.*[1] suggested that the effects of lead may be mainly on the cellular immune system. Results of the *in vitro* studies included in the review showed an inhibition of T-helper 1 cell activation and enhancement of T-helper 2 dell activation. T-helper 1 cells produce IFN-γ, while T-helper 2 cells produce TNF, IL-6, and IL-10. Our results agree with the premise that the T helper cell lines are affected differently by lead. Both TNF-α and IL-6 showed a statistically significant dose-dependent increase in production.

The influence of modifying factors on the cellular immune response these chemicals must, however, be given consideration. As shown in this study, duplication of trends of *h*-PBMC cytokine production cannot be achieved unequivocally despite strict adherence to the experimental protocol. Sleep deprivation,[18] aging,[19] and stress,[20] which are among the factors shown to modulate cytokine production, may have affected the experimental outcome.

Nonetheless, the differential effects of heavy metal exposure on the cytokine profile of *h*-PBMCs were demontrated by the results of our experiment. Cytokines may be potential biomarkers to elucidate the mechanisms of heavy metal immunotoxicity. Metal-induced immunomodulation at concentrations that do not affect cell viability may have important implications in establishing acceptable levels of human exposure to the heavy metals used. Although intra- and interindividual variability has been observed in the present study and by comparing the results of the present to previous ones, the value of cytokines in effect monitoring deserves investigation. Further study of the implications of *in vitro* experiments on the cytokine profile of heavy metal-exposed cells is needed to extrapolate the results to human situations.

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