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INORGANIC COMPOUNDS

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# Oral exposure to inorganic mercury alters T lymphocyte phenotypes and cytokine expression in BALB/c mice

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**Abstract** Mercury is a well-recognized health hazard and an environmental contaminant. Mercury modulates immune responses ranging from immune suppression to autoimmunity but the mechanisms responsible for these effects are still unclear. Male BALB/c mice were exposed continuously to 0, 0.3, 1.5, 7.5, or 37.5 ppm mercury in drinking water for 14 days. Body weight was reduced at the highest dose of mercury whereas the relative kidney and spleen weights were significantly increased. The dose range of mercury used did not cause hepatotoxicity as indicated by circulating alanine aminotransferase and aspartate aminotransferase levels. Circulating blood leukocytes were elevated in mice treated with the highest dose of mercury. Mercury ranging from 1.5 to 37.5 ppm dose-dependently decreased $CD3^+$ T lymphocytes in spleen; both $CD4^+$ and $CD8^+$ single-positive lymphocyte populations were decreased. Exposure to 7.5 and 37.5 ppm mercury decreased the $CD8^+$ T lymphocyte population in the thymus, whereas double-positive $CD4^+/CD8^+$ and $CD4^+$ thymocytes were not altered. Mercury altered the expression of inflammatory cytokines (tumor necrosis factor $\alpha$, interferon $\gamma$, and interleukin-12), c-myc, and major histocompatibility complex II, in various organs. Results indicated that a decrease in T lymphocyte populations in immune organs and altered cytokine gene expression may contribute to the immunotoxic effects of inorganic mercury.

**Keywords** Mercury · T lymphocytes · Drinking water · Inflammatory cytokines · Major histocompatibility complex II · Immunotoxicity

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## Introduction

Mercury is widespread in the environment, and chronic exposure to low levels of mercury is possible due to contamination of food and drinking water supplies. Mercury is highly toxic, and moderate levels of exposure to mercury can cause neurotoxic manifestations, nephrotoxicity, immune function alterations, and decreased host resistance to viral infections in mice (Christensen et al. 1996; Gerstner and Huff 1977; Wild et al. 1997). Mercury exists in different forms, e.g., elemental mercury, and inorganic and organic mercury compounds. They have some properties in common but differ in their metabolism and toxicity. The distribution of mercury within the body and in specific organs varies with the chemical form, the dose and the time after exposure (Cember et al. 1968). Additionally, the route of administration affects the organ distribution of absorbed mercury (Nielsen and Andersen 1989, 1990). Although the use of inorganic mercury compounds has decreased in recent years and precautions against industrial emissions have been increased, future human exposure to inorganic mercury will probably result in a few individuals, including those occupationally exposed, and populations exposed to low levels from dental amalgam or from food or drinking water containing mercury. Parenteral administration of soluble mercury salts has been the commonly used exposure route in past animal studies, despite natural human exposure being via the oral route, i.e., from drinking water and food (Nielsen and Andersen 1989).

Recently, there has been growing concern regarding the effects on human health of low level of environmental mercury contamination (Kaiser 2000). Although mercury is broadly sulfhydryl reactive, surprisingly little is understood as to the specific cellular targets and/or biochemical pathways targeted by this metal. Clinical studies on industrial workers exposed to mercury levels within the World Health Organization guidelines have shown immune system abnormalities in the absence of gross neurological dysfunction (Perlingeiro and Queiroz 1994). The immune system is probably an important target organ for mercury intoxication as well as a particularly revealing sentinel system in which to investigate the biochemical effects of low-level mercury exposure. Prolonged exposure of mice to relatively low concentrations of mercury increased susceptibility to a variety of viruses (Koller 1975). Mercury exposure was shown to increase serum immunoglobulin $G_1$ ($IgG_1$) and IgE levels in certain strains of mice or rats after subcutaneous injection (Pietsch et al. 1989; Prouvost-Danon et al. 1981), and caused autoantibody formation to glomerular basement membrane in rats (Sapin et al. 1977). We recently reported that noncytotoxic concentrations of inorganic mercury inhibited nitric oxide (NO) production and altered proinflammatory cytokine gene expressions in murine macrophages in vitro (Kim et al. 2002). Mercury impaired host defense to bacterial infection via decreasing NO production. Mercury also altered production of various cytokines in both human and murine lymphocytes (Jiang and Moller 1995; Shenker et al. 1992), such as interleukin-1 (IL-1), IL-2, IL-4, and interferon $\gamma$ (IFN$\gamma$), and proto-oncoprotein c-myc (Badou et al. 1997; Hu et al. 1997; Johansson et al. 1997; Shenker et al. 1992; Vamvakas et al. 1993).

Because of the public health importance of mercury in the environment and its potential effect on the immune system, it is desirable to further characterize the effect of this system. Inorganic mercury has been implicated in the production of autoimmune responses in susceptible strains of mice (Hultman et al. 1993). BALB/c mice did not produce antichromatin and antihistone antibodies, as observed in SJL/J and other responding strains; however, the formation of renal mesangial and vessel wall immune deposits were observed in BALB/c strain in response to inorganic mercury. Hu et al. (1997) reported that mercuric chloride produced major histocompatibility comlex class II (MHC II)-dependent induction of various cytokines such as IL-2, IL-4 and IFN$\gamma$ in murine splenocytes in vitro. Previous immunotoxicity studies with mercury have primarily investigated alterations in immunoglobulins and development of autoimmunity (Pietsch et al. 1989; Hultman et al. 1992). Little is known about specific lymphocyte populations and immune modulation in peripheral organs after mercury treatment. Therefore, the current study was undertaken to investigate the effects of mercury on the populations of different lymphocytes in lymphoid organs. The expression of MHC II and dependent cytokines in major immune organs (spleen and thymus) and in other target organs for this metal, e.g., liver and kidney were also determined. Representative proinflammatory cytokine such as tumor necrosis factor $\alpha$ (TNF$\alpha$) and an anti-inflammatory cytokine, transforming growth factor $\beta$ (TGF$\beta$), were also evaluated along with the expression of pro-apoptotic factor c-myc in immune organs. We hypothesized that the observed immunotoxic effects of low levels of mercury are induced by alterations in cellular subpopulations in immune organs, and the toxic response in organs is reflected by the modulation of major cytokines. Different cytokines were selected on the basis of their inflammatory or proliferative potential. Male BALB/c mice were treated via drinking water, a relevant route of mercury exposure, to determine these responses to mercury. In addition to the immune organs, i.e., spleen and thymus, we investigated the proinflammatory cytokine production in liver and kidney, the organs that have a local network of immunologic responses.

## Materials and methods

### Animal care and handling

Inbred male BALB/c mice (specific-pathogen free, Harlan Inc., Indianapolis, IN, USA), 6 weeks of age and with an average body weight of 20 g, were procured. Mice were randomly assigned to treatment groups (four per cage) and acclimatized for 1 week in the housing facility maintained at 21°C with a 12 h/12 h light/dark cycle. The mice were housed in polycarbonate shoebox-style cages lined with wood chip bedding (Betachip, Northeastern Products Corporation, Warrensberg, NY, USA), which was changed every third day. Rodent chow, Harlan Teklad 22/5 rodent chow (Harlan Teklad, Madison, WI, USA), and purified water (Milli-Q Water Purification System, Millipore, Bedford, MA, USA) were supplied ad libitum. Food and water consumption, as well as bodyweight gain, were recorded daily. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

### Treatment

Mercuric chloride (Sigma, St. Louis, MO, USA) was administered in the purified drinking water as 0, 0.3, 1.5, 7.5, or 37.5 mg/l (ppm) mercury. Mice were continuously provided the mercury-containing water for 14 days; the water was replaced every other day with freshly prepared solutions. At the end of treatment period, mice were fasted overnight and killed by decapitation. Trunk blood was collected, and spleen, thymus, liver, and kidneys were aseptically excised and weighed.

### Hematology and estimation of liver enzymes in plasma

Total blood erythrocyte and leukocyte counts were determined using an electronic counter (Coulter, Hialeah, FL, USA). Levels of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a Hitachi 912 autoanalyzer (Roche Diagnostics, Indianapolis, IN, USA).

### Preparation of single-cell lymphocyte populations

Single-cell lymphocyte populations were prepared from spleen and thymus as described earlier (Johnson and Sharma 2001) with modifications as noted below. Organs were maintained in 10 ml of cold complete RPMI medium [RPMI 1640 (Gibco–Life Technol-

ogies, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% Pen-Strep (penicillin-streptomycin; Gibco)]. Monocellular suspensions were prepared using a Stomacher laboratory blender (STOM 80; Seward, Thetford, UK), and connective tissue was removed by passing through a 120 μm screen. The cell suspension was washed twice with RPMI for 10 min at room temperature. The cell pellet was resuspended in complete RPMI medium, and lymphocyte cell counts performed using a hemocytometer. Cell viability was determined using the Trypan blue (Gibco) exclusion and was greater than 95%.

### Flow cytometry phenotyping of splenic and thymic lymphocyte populations

Three-color flow cytometry was used to determine the prevalence of specific lymphocyte populations in the thymus and spleen. Monoclonal antibodies were conjugated to fluorescein isothiocyanate (FITC, emission at 525 nm), R-phycoerythrin (R-PE, emission at 575 nm) or TRI-COLOR (PE-Cy5 tandem transfer dye, emission at 650 nm). Antibodies to cell-specific receptors (Caltag Laboratories, Burlingame, CA, USA) included hamster anti-mouse CD3-FITC (pan T-lymphocyte), rat anti-mouse CD45/B220-R-PE (pan B-lymphocyte), rat anti-mouse CD4-R-PE (T-helper lymphocyte) and rat anti-mouse CD8-TRI-COLOR (T-cytotoxic/suppressor lymphocyte). Mouse anti-mouse CD32/16 antibodies were used to block non-specific binding of Fc$\gamma$ II/III receptors. Cells were washed in phosphate-buffered saline (PBS), and antibodies were added to samples and vortexed gently, followed by incubation at 4°C for 30 min. Cells were washed three times in PAB (PBS with 1% bovine serum albumin and 0.1% $NaN_3$) and then resuspended in 0.5% formalin in PBS while vortexing. Fixed cells were maintained at 4°C in the dark until acquisition ( < 24 h). Cells were acquired (20,000 events) using an EPICS XL-MCL flow cytometer (Coulter) equipped with a 488 nm argon ion laser and Lysis II acquisition software. Analysis was performed using WinMDI flow analysis software.

### RNA isolation and semi-quantitative estimation of cytokine expression

RNA was isolated from the tissue using the protocol described earlier (Tsunoda and Sharma 1999). First-strand complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Life Technologies, Grand Island, NY, USA). Reverse-transcriptase–polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF$\alpha$, IFN$\gamma$, IL-1$\beta$, IL-4, IL-12, MHC II, c-myc, TGF$\beta$, and $\beta$-actin (internal control). The conditions for RT-PCR were as previously reported (Kim et al. 2002; Tsunoda and Sharma 1999). Cycle number was optimized (26–40 cycles based on preliminary trials) to ensure product accumulation in an exponential range. Amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide. The gels were documented using a Kodak DC 290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). Band intensities for the genes of interest were normalized to that of $\beta$-actin in the same sample.

### Statistical analysis

All statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A value of $P < 0.05$ was used to indicate significant differences.

## Results

Food and water consumption decreased in a dose-dependent manner in mice exposed to mercury in drinking water. Exposure to 0.3 or 1.5 ppm mercury as mercuric chloride did not alter the water consumption, but mercury at 7.5 or 37.5 ppm decreased daily water consumption by 13.9 and 40.5%, respectively, compared with the control values. Food consumption was decreased by 14.4% in mice treated with 37.5 ppm mercury (Table 1). Mercury affected bodyweight gain and relative organ weights (Table 2). Mice treated with 37.5 ppm mercury for 14 days exhibited 2.1% reduction in bodyweight compared with an 8.0% gain in the control mice. However, the relative kidney and spleen (organ/body weight ratios) weights were significantly

**Table 1** Mercury exposure levels and water and food consumption in male BALB/c mice. Means ± SE ($n = 4$)

| Mercury in water (ppm) | Dose of mercury (mg/kg per day)[a] | Water consumption (ml/day per group) | Food consumption (g/day per group) |
|---|---|---|---|
| 0 | 0 | 19.57 ± 0.86 | 13.84 ± 0.77 |
| 0.3 | 0.06 | 18.50 ± 0.81 | 14.21 ± 0.46 |
| 1.5 | 0.31 | 19.14 ± 1.14 | 14.45 ± 0.59 |
| 7.5 | 1.39 | 16.85 ± 0.90* | 13.45 ± 1.58 |
| 37.5 | 4.81 | 11.64 ± 1.26* | 11.85 ± 0.45* |

[a]Received dose calculated on the basis of water consumption
*$P < 0.05$, significantly different from the control group (0 ppm mercury) values

**Table 2** Effects of oral exposure to mercuryt for 14 days on bodyweight gain and relative organ weights of male BALB/c mice. Means ± SE ($n = 4$)

| Mercury in water (ppm) | Bodyweight gain (g) (% change from initial value) | Organ weight/body weight ratio (g/100 g) for | | | |
|---|---|---|---|---|---|
| | | Liver | Kidney | Spleen | Thymus |
| 0 | 1.72 ± 0.51 (8.0%) | 4.70 ± 0.08 | 1.65 ± 0.02 | 0.31 ± 0.01 | 0.21 ± 0.04 |
| 0.3 | 1.72 ± 0.24 (8.6%) | 4.59 ± 0.11 | 1.62 ± 0.01 | 0.35 ± 0.02 | 0.27 ± 0.02 |
| 1.5 | 1.65 ± 0.27 (7.9%) | 4.72 ± 0.04 | 1.64 ± 0.04 | 0.34 ± 0.01 | 0.27 ± 0.01 |
| 7.5 | 1.45 ± 0.19 (7.1%) | 4.70 ± 0.04 | 1.83 ± 0.01* | 0.38 ± 0.02 | 0.25 ± 0.01 |
| 37.5 | −0.45 ± 0.43 (−2.1%)* | 4.78 ± 0.17 | 2.05 ± 0.06* | 0.40 ± 0.02* | 0.26 ± 0.01 |

*$P < 0.05$, significantly different from the control group (0 ppm mercury) values

increased in mice exposed to 37.5 ppm mercury. The relative liver and thymus weights were unaltered.

Hematological parameters were affected by mercury exposure. Total erythrocyte counts were significantly decreased at all concentration of mercury treatment (Table 3). Leukocyte counts were increased at the highest dose of mercury. Oral treatment with mercury did not change plasma ALT and AST levels, suggesting a lack of overt hepatotoxicity.

Exposure to mercury by drinking water dose-dependently decreased specific lymphocyte populations in spleen and thymus of mice. Oral exposure to mercury at 1.5, 7.5, and 37.5 ppm decreased $CD3^+$ T lymphocytes in the spleen by 14.1, 20.5, and 25.4%, respectively, whereas population of $CD45^+$ B lymphocytes remained unchanged (Table 4). Dose-dependent decreases in both $CD4^+$ and $CD8^+$ T lymphocyte populations in the spleen paralleled the decreases in the total $CD3^+$ T lymphocyte population. Oral exposure to 7.5 and 37.5 ppm mercury decreased the population of $CD8^+$ cytolytic T lymphocytes in thymus by 46.9 and 50.0%, respectively (Table 5). The double-positive $CD4^+/CD8^+$ and the single-positive $CD4^+$ thymocyte populations did not decrease significantly.

In the spleen, a peripheral immune organ, oral exposure to mercury decreased expression of IFN$\gamma$ and MHC II in a dose-dependent manner (Fig. 1). At the highest dose of mercury, the expression of c-myc was increased. Expression of other cytokines in the spleen, such as TNF$\alpha$, IL-1$\beta$, IL-4, IL-12, and TGF$\beta$, were not altered by mercury. In the thymus, the results were somewhat diverse. Expression of TNF$\alpha$, IL-12, and c-myc were significantly increased by mercury treatment at the higher doses (Fig. 2). Mercury decreased the expression for MHC II at the highest dose. Expression of other cytokines measured in the thymus, i.e., IFN$\gamma$, IL-1$\beta$, IL-4, and TGF$\beta$, were not altered by mercury treatment.

**Table 3** Effects of oral exposure to mercury for 14 days on circulating erythrocyte and leukocyte counts, and liver enzymes in male BALB/c mice. Means ±SE ($n=4$). *RBC* red blood cells, *WBC* white blood cells, *ALT* alanine aminotransferase activity, *AST* aspartate aminotransferase activity

| Mercury in water (ppm) | RBC ($\times10^{-6}$/mm$^3$) | WBC ($\times10^{-3}$/mm$^3$) | ALT (U/l) | AST (U/l) |
|---|---|---|---|---|
| 0 | 7.61 ± 0.20 | 2.20 ± 0.19 | 38.25 ± 3.09 | 215.25 ± 31.76 |
| 0.3 | 6.64 ± 0.32* | 2.70 ± 0.57 | 38.75 ± 0.75 | 228.75 ± 7.90 |
| 1.5 | 6.61 ± 0.13* | 3.30 ± 0.65 | 40.25 ± 4.11 | 201.75 ± 26.85 |
| 7.5 | 6.75 ± 0.08* | 2.53 ± 0.50 | 40.00 ± 6.46 | 217.75 ± 52.89 |
| 37.5 | 6.15 ± 0.23* | 4.21 ± 0.76* | 29.50 ± 2.53 | 143.25 ± 22.96 |

*$P<0.05$, significantly different from the control (0 ppm mercury)

**Table 4** Effects of oral exposure to mercury for 14 days on lymphocyte populations in the spleen of male BALB/c mice. Populations are represented as absolute cell numbers expressing a given receptor. Means ±SE ($n=4$)

| Mercury in water (ppm) | Splenocytes ($\times10^{-5}$) per spleen expressing receptor | | | | $CD4^+/CD8^+$ Ratio |
|---|---|---|---|---|---|
| | $CD3^+$ (T lymphocytes) | $CD45/B220^+$ (B lymphocytes) | $CD4^+$ (T-helper) | $CD8^+$ (T-suppressor/cytotoxic) | |
| 0 | 47.69 ± 0.93 | 52.80 ± 1.04 | 31.29 ± 0.64 | 14.08 ± 0.27 | 2.22 ± 0.04 |
| 0.3 | 43.59 ± 0.64 | 53.87 ± 2.30 | 28.75 ± 0.55 | 12.93 ± 0.53 | 2.22 ± 0.07 |
| 1.5 | 41.01 ± 1.65* | 53.44 ± 1.25 | 26.92 ± 1.20* | 12.02 ± 0.53* | 2.24 ± 0.03 |
| 7.5 | 37.91 ± 1.81* | 55.52 ± 1.81 | 25.04 ± 1.30* | 10.89 ± 0.58* | 2.30 ± 0.06 |
| 37.5 | 35.56 ± 0.94* | 58.81 ± 2.63 | 23.14 ± 0.61* | 10.69 ± 0.35* | 2.16 ± 0.04 |

*$P<0.05$, significantly different from the control group (0 ppm mercury)

**Table 5** Effects of oral exposure to mercury for 14 days on lymphocyte populations in the thymus of male BALB/c mice. Populations are represented as absolute cell numbers expressing a given receptor. Means ±SE ($n=4$)

| Mercury in water (ppm) | Thymocytes ($\times10^{-5}$) per thymus expressing receptor | | |
|---|---|---|---|
| | $CD4^+/CD8^+$ (double-positive) | $CD4^+/CD8^-$(helper) | $CD4^-/CD8^+$(suppressor/cytotoxic) |
| 0 | 63.35 ± 4.94 | 8.58 ± 0.82 | 2.81 ± 0.29 |
| 0.3 | 56.54 ± 6.71 | 7.06 ± 1.06 | 2.08 ± 0.29 |
| 1.5 | 54.08 ± 7.21 | 6.63 ± 0.82 | 1.98 ± 0.23 |
| 7.5 | 63.02 ± 2.92 | 6.56 ± 0.43 | 1.49 ± 0.15* |
| 37.5 | 59.23 ± 6.21 | 6.05 ± 0.69 | 1.38 ± 0.16* |

*$P<0.05$, significantly different from the control group (0 ppm mercury)

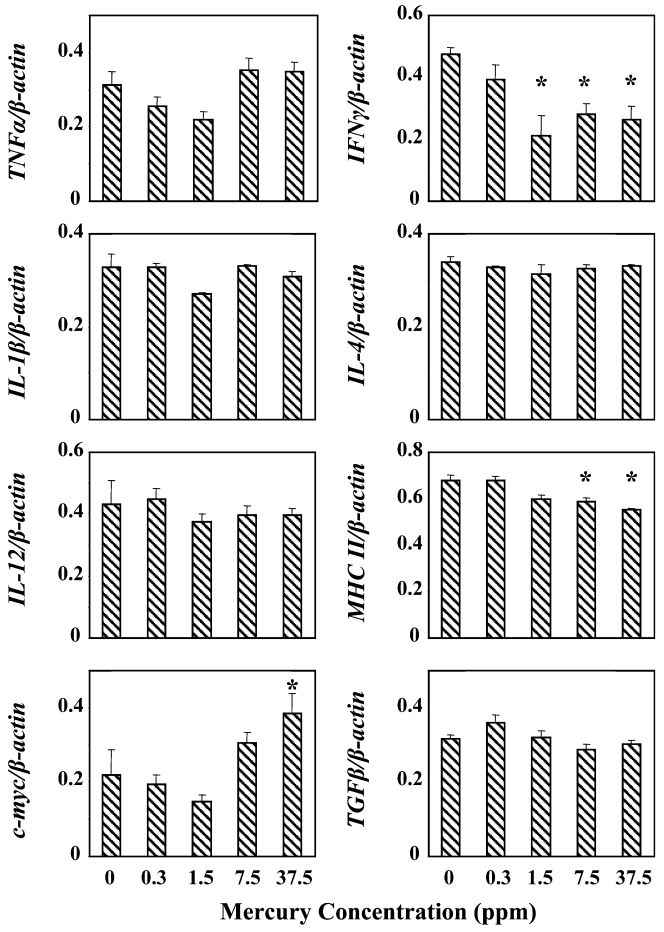


**Fig. 1** The effect of inorganic mercury on the expression of various cytokines and other factors in spleen. Male BALB/c mice were treated with 0.3, 1.5, 7.5, or 37.5 ppm mercury in the drinking water for 14 days. Extraction and analysis of mRNA was performed as described under Materials and methods section. Gene expression was analyzed by RT-PCR. Results are expressed relative to $\beta$-actin as means $\pm$SE ($n=4$) (*TNF*$\alpha$ tumor necrosis factor $\alpha$, *IFN*$\gamma$ interferon $\gamma$, *IL-1*$\beta$ interleukin-1$\beta$, *IL-4* interleukin-4, *IL-12* interleukin-12, *MHC II* major histocompatibility complex II, *c-myc* proto-oncoprotein c-myc, *TFG*$\beta$ transforming growth factor $\beta$). *$P<0.05$, significantly different from the control group values

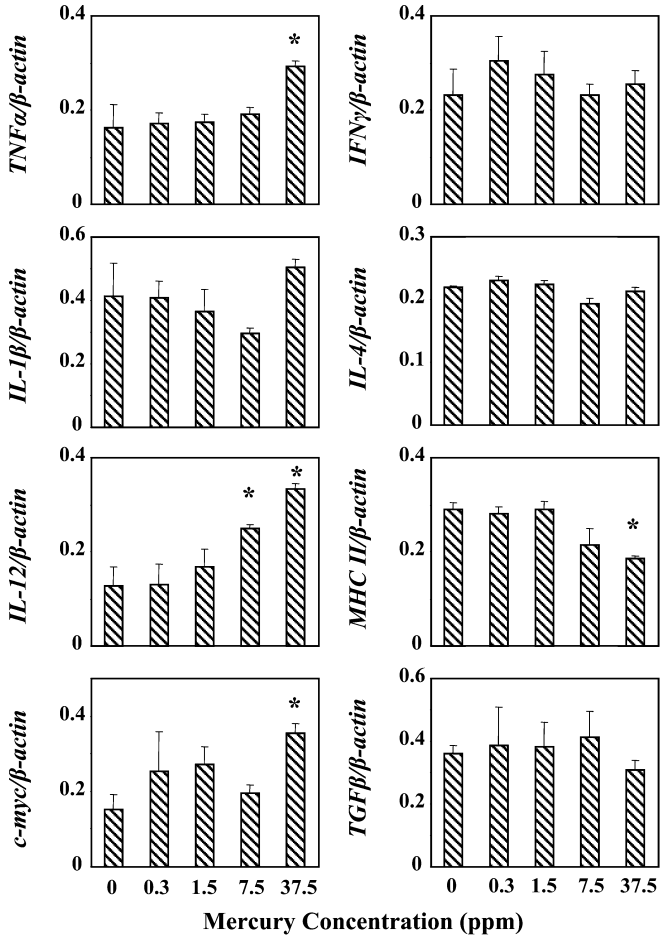


**Fig. 2** The effect of inorganic mercury on the expression of cytokines and related factors in thymus. Male BALB/c mice were treated with 0.3, 1.5, 7.5, or 37.5 ppm mercury in the drinking water for 14 days. Extraction and analysis of mRNA was performed as described under Materials and methods section. Gene expression was analyzed by RT-PCR. Results are expressed relative to $\beta$-actin as means $\pm$SE ($n=4$) (*TNF*$\alpha$ tumor necrosis factor $\alpha$, *IFN*$\gamma$ interferon $\gamma$, *IL-1*$\beta$ interleukin-1$\beta$, *IL-4* interleukin-4, *IL-12* interleukin-12, *MHC II* major histocompatibility complex II, *c-myc* proto-oncoprotein c-myc, *TFG*$\beta$ transforming growth factor $\beta$). *$P<0.05$, significantly different from the control group values

The cytokine expression in liver and kidney, target organs for mercury toxicity, was different compared with that observed for spleen and thymus. In liver, oral exposure to mercury increased the expression of TNF$\alpha$, IFN$\gamma$, IL-12, and MHC II (Fig. 3). Other pro-inflammatory cytokines IL-1$\beta$ and IL-4 were not changed following mercury exposure. Changes in expression of various cytokines in the kidney were similar to that observed in the liver. Exposure to 37.5 ppm mercury significantly increased expression of TNF$\alpha$, IFN$\gamma$, IL-12, and MHC II (Fig. 4). Expression of IL-1$\beta$, and IL-4 in kidney was not altered by mercury exposure at the doses employed.

## Discussion

The exposure of BALB/c mice to mercury for 14 days via drinking water caused marginal toxicity other than that observed in the parameters of the immune system. The decrease in erythrocyte counts may have been related to a decrease in water consumption, whereas increase in leukocyte counts and weights of some organs at the higher doses of mercury exposure reflects inflammatory responses. Hepatotoxicity was not evident by circulating levels of liver enzymes; nephrotoxicity was reflected by increased kidney weights. No other tests for nephrotoxicity, a well-known response to low levels of mercury, were performed in these trials.

Results of this study indicated that exposure of BALB/c mice to inorganic mercury via drinking water caused immunologic alterations, i.e., changes in T lymphocyte phenotypes and cytokine expression, and in hematological parameters. Our current data showing a decrease in the $CD3^+$ T lymphocyte population and,

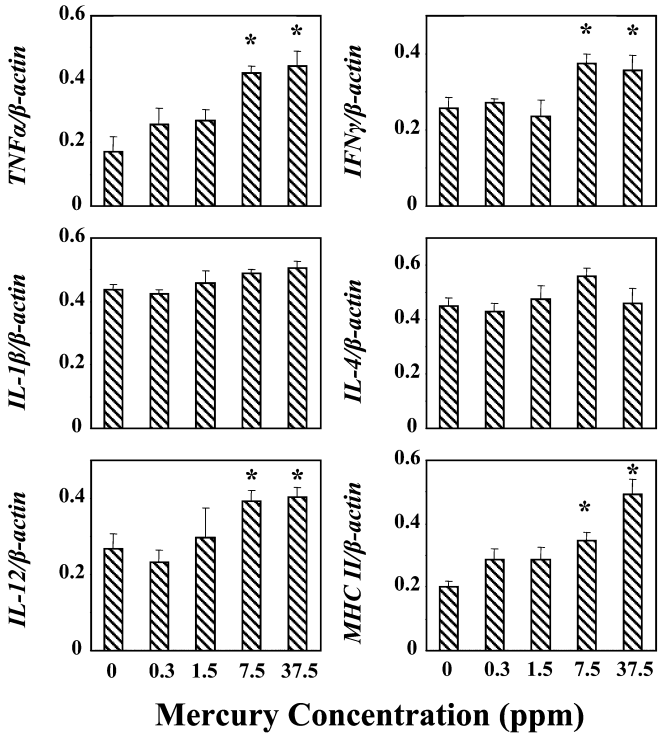


**Fig. 3** The effect of inorganic mercury on the expression of cytokines and related factors in liver. Male BALB/c mice were treated with 0.3, 1.5, 7.5, or 37.5 ppm mercury in the drinking water for 14 days. Extraction and analysis of mRNA was performed as described under Materials and methods section. Gene expression was analyzed by RT-PCR. Results are expressed relative to $\beta$-actin as means $\pm$SE ($n=4$) (*TNF*$\alpha$ tumor necrosis factor $\alpha$, *IFN*$\gamma$ interferon $\gamma$, *IL-1*$\beta$ interleukin-1$\beta$, *IL-4* interleukin-4, *IL-12* interleukin-12, *MHC II* major histocompatibility complex II). *$P<0.05$, significantly different than the control group values

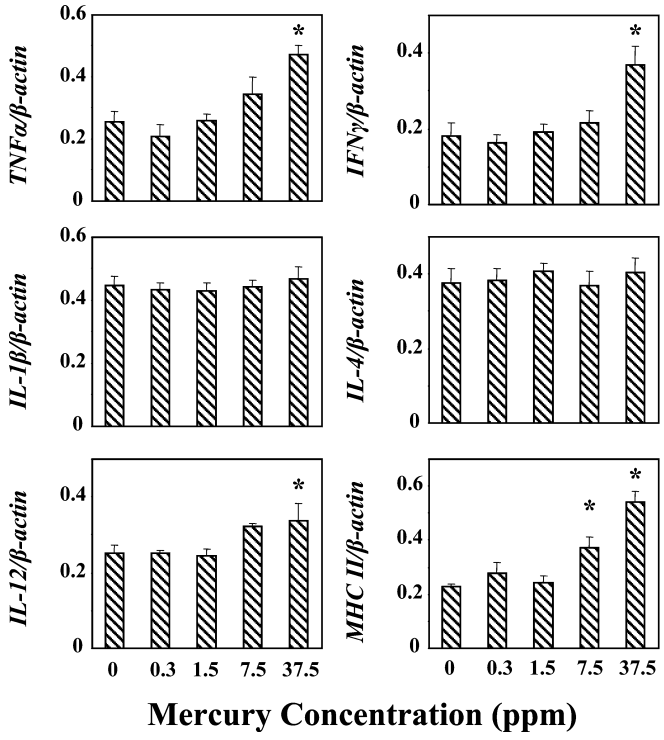


**Fig. 4** The effect of inorganic mercury on the expression of cytokines and other factors in kidney. Male BALB/c mice were treated with 0.3, 1.5, 7.5, or 37.5 ppm mercury in the drinking water for 14 days. Extraction and analysis of mRNA was performed as described under Materials and methods section. Gene expression was analyzed by RT-PCR. Results are expressed relative to $\beta$-actin as means $\pm$SE ($n=4$) (*TNF*$\alpha$ tumor necrosis factor $\alpha$, *IFN*$\gamma$ interferon $\gamma$, *IL-1*$\beta$ interleukin-1$\beta$, *IL-4* interleukin-4, *IL-12* interleukin-12, *MHC II* major histocompatibility complex II). *$P<0.05$, significantly different from the control group values

among the T lymphocytes, both $CD4^+$ and $CD8^+$ T lymphocytes without a change in the number of B lymphocytes in spleen, are consistent to an early report in rats. A 14-day of treatment with inorganic mercury altered cervical lymph node lymphocyte phenotypes in Brown Norway rats (Kosuda et al. 1998). In mercury-susceptible Brown Norway rats, mercury decreased $CD4^+$ and $CD8^+$ populations without changing the population of B cells or $CD4^+/CD8^+$ double-positive T lymphocytes. No changes were seen in the ratio of $CD4^+$ and $CD8^+$ T lymphocytes, which indicated that the general impact of mercury on T lymphocyte is not a skewing of the subtype.

Mercury elicits a spectrum of immunologic responses ranging from immunosuppression to autoimmunity (Koller 1980). Administration of 14.8 mg mercury/kg per day as mercuric chloride to B6C3F mice for 2 weeks showed immune suppression, including a decrease in thymus weight (National Toxicology Program 1993). Mercury decreased proliferation of $CD4^+$ lymph node T lymphocytes, and IL-3 and IFN$\gamma$ secretion, leading to apoptotic cell death (Shen et al. 2001). In the present study, it is tempting to speculate that decrease in specific types of lymphocytes was not likely to be due to mercury-induced apoptosis in peripheral lymphoid organs because mercury did not decrease the weight of thymus and increased the weight of spleen; moreover, the number of apoptosis-sensitive double-positive T lymphocyte in thymus remained unchanged. Induction of c-myc in spleen, and TNF$\alpha$ and c-myc in thymus, however, are suggestive of the onset of apoptotic process. Earlier, mercury caused thymus atrophy and changes in thymocyte subpopulations, but the atrophy of thymus was reported not to be due to the apoptotic effect of mercury (Kosuda et al. 1996).

The expression of TGF$\beta$ was not altered by mercury treatment although TGF$\beta$ is a crucial regulatory cytokine that abolishes T cell activation and causes pathogenic situations such as allergic diseases and autoimmune diseases. The mercury-induced alteration of lymphocyte populations in spleen and thymus is likely to be due to a TGF$\beta$-independent pathway or the activation of Smad binding proteins. Kunzmann et al. (2003) recently reported that Smad binding proteins attenuated TGF$\beta$ signaling in $CD4^+$ T lymphocytes. The activation level of T cells influenced the expression of Smad binding proteins and the response to TGF$\beta$.

In our present study, relatively high concentrations of mercury suppressed gene expression of MHC II, which is important for T lymphocyte-mediated immune responses. These results are consistent with a decrease of $CD4^+$ and $CD8^+$ lymphocyte populations, and a decrease in IFN$\gamma$, which is related to T lymphocyte-mediated activation of macrophages. The present data are in accordance with previous reports showing increased expression of proto-oncogene c-myc after mercury treatment, which is known to be proliferative and also apoptotic (Vamvakas et al. 1993). Decreased expression of MHC II and IFN$\gamma$ in spleen may reflect the reduced lymphocyte subpopulations. It is likely that depletion of selected phenotypes from spleen and thymus followed their translocation to organs like liver and kidney, and thereby produced the downregulation of cytokines in these immunologic organs. Instead of immunosuppressive effects, intoxication by mercury stimulated localized immune responses in liver and kidney, suggesting mild inflammatory changes and resulting increased cytokine expression. In both liver and kidney, relatively high concentrations of mercury increased expressions of TNF$\alpha$, IL-12, IFN$\gamma$, and MHC II. In previous reports cytokines such as TNF$\alpha$, IL-1, IL-4, IL-12, and IFN$\gamma$ were induced after in vivo or in vitro mercury treatments and may have contributed to the immunologic and cell proliferative responses (Johansson et al. 1997; Pollard and Hultman 1997; Reardon and Lucas 1987). Madrenas et al. (1991) reported that mercury increased renal MHC II expression and treatment of anti-IFN$\gamma$ antibody blocked the MHC II induction. In this study the mercury-induced MHC II induction was mediated by IFN$\gamma$ in the renal system and supports our present results showing increases of both IFN$\gamma$ and MHC II in kidney.

The cytokine network is dependent on the expression of different cytokines that induce or suppress the production of other cytokines. IL-12 is a proinflammatory cytokine that induces IFN$\gamma$ and helps the differentiation of T-helper 1 (Th1) cells (Trinchieri 2003). Treatment of mouse fetal thymic organ culture with IL-12 caused a significant increase in both the percentage and number of $CD8^+$ thymocytes (Godfrey et al. 1994). Because $CD8^+$ thymocytes were significantly decreased by mercury, IL-12 may be produced in response to the stimulation of thymus to differentiate $CD8^+$ lymphocytes. IFN$\gamma$ upregulates MHC II (Blanck 2002), and palys a role in Th1-mediated autoimmunity; however, paradoxical effects of IFN$\gamma$ in models of autoimmunity have been suggested (Rosloniec et al. 2002). MHC II is also involved in both cytokine production and proliferation (Hu et al. 1997). IFN$\gamma$ is an important cytokine that stimulates macrophages, and MHC II promotes the ability of stimulated macrophages to serve as antigen-presenting cells to T lymphocytes (Locksley et al. 1995). The involvement of MHC II suggests that the two cell types, T cells and macrophages, are required for polyclonal proliferation, and may contribute to mercury-induced immunologic responses, including autoimmunity (Johansson et al. 1997; Pollard and Hultman 1997).

It should be emphasized that the changes reported here were measured after a 2-week treatment with mercury. Cytokine changes may occur early during the treatment period and it is desirable to investigate the time-related alterations in cytokine expression. Additionally, it is not clear whether the effects observed were direct or indirect effects of mercury. The kinetics of cytokine expression and detailed effects of mercury on immune responses need to be evaluated in future studies.

Because of a broad range of immunomodulant properties, mercury is considered as paradigm of xenobiotic immunotoxicity, and may be a useful tool for investigation of the cytokine network and as a model for autoimmune disease (Bigazzi 1998). Our present data suggest that mercury exposure via contaminated drinking water may alter lymphocyte subpopulations in peripheral lymphoid T lymphocyte and modulate cytokine expression in various organs. The molecular mechanisms of mercury-induced immunomodulation remain to be elucidated. To the best of our knowledge this is a first report of investigating the cytokine network in liver and kidney with low levels of mercury exposure.

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