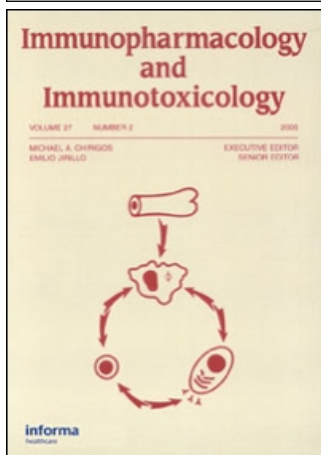


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Selective Effect of Mercury on Th2-Type Cytokine Production in Humans

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Mercury induces autoimmune disease and increases IL-4 production and IgE levels in certain rodent strains. The object of this study was to determine if mercury was capable of inducing Th2 cytokine production in human leucocytes. Human peripheral blood mononuclear cells (PBMC) were incubated with PMA/ionomycin or Con A in the presence or absence of methyl mercury (CH_3Hg) or mercuric chloride (HgCl_2). IL-4 and gamma-IFN were measured by ELISA. **RESULTS:** IL-4 production significantly increased at low concentrations of CH_3Hg (0.5 μM , $p < 0.01$), while gamma-IFN production was suppressed starting at CH_3Hg 2 μM ($p = 0.004$). Inorganic mercury (HgCl_2) increased IL-4 only at concentrations 10–20 times higher than CH_3Hg . These findings suggest a mechanism by which mercury could trigger or potentiate TH2 cytokine production in humans.

Keywords Mercury, Methyl Mercury, Mercuric Chloride, Cytokine, IL-4, Gamma-IFN.

INTRODUCTION

Mercury levels have been increasing in the environment over the past 50 years.⁽¹⁾ A major source of human exposure to mercury, in the form of methyl mercury, is from the consumption of contaminated fish.⁽²⁾ Mercury is liberated into the biosphere mainly by burning fossil fuel and once settled into lakes or rivers it can be converted into methyl mercury by microorganisms that are in turn consumed by fish. Dental amalgam fillings and vapors are another source of mercury exposure.^(3,4) However, while methyl mercury originating

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from fish is nearly 100% absorbed, only approximately 10–20% of swallowed mercury ions is absorbed in the gastrointestinal tract,⁽⁵⁾ although methylation may occur within the body. Increased mercury levels in fish and concern about their deleterious health effects have led U.S. public health authorities to warn pregnant women to minimize their fish consumption, especially of those like swordfish that contain very high levels of mercury.

Although most of the health concerns about mercury are related to its toxic effects on the nervous system and kidney, a number of studies in experimental animals indicate that mercury can also promote autoimmune disease at subtoxic levels, by a TH2 driven mechanism. Serum IgE levels increase dramatically in genetically susceptible rats given experimental mercury amalgam fillings⁽⁶⁾ and mercuric chloride (HgCl₂) treated rats or mice exhibit increased plasma IL-4 levels^(7,8) as well as increased IL-4 mRNA in lymphoid tissue.^(9–10) The generation of gamma-IFN producing cells in these animals was decreased.^(11,12) Murine mast cells were shown to produce IL-4 and TNF-alpha when exposed to HgCl₂ *in vitro*⁽¹³⁾ and splenic cells from mice that were injected with HgCl₂ exhibited a significant increase in the IL-4: gamma-IFN ratio when stimulated with anti-CD3 compared to the control group.⁽⁶⁾

The effect of mercury on immunologic dysfunction in humans has been less well studied. However, an association between elevated urine mercury levels in children and acute atopic eczema and increased serum IgE levels was recently reported.⁽¹⁴⁾ Another recent study found an association between mercury exposure and autoimmune markers such as serum antinuclear antibodies (ANA) and antinucleolar antibodies.⁽¹⁵⁾ In another study, decreased CD4+CD45RA (naïve) T-cells and T-cell proliferation was found in children exposed to polychlorinated biphenyls and methyl mercury through fish-consumption in a maritime population.⁽¹⁶⁾ In this latter study, mercury was inversely correlated to plasma IgM. Relatively little is known about potential mechanisms of how mercury affects the human immune system, although Strenzke et al. have shown that mercury increases IL-4, LTC₄ and histamine production in basophils when co-incubated with anti-IgE.⁽¹⁷⁾

IgE production is predominantly triggered by TH2 cytokines such as IL-4 and is suppressed by TH1 cytokines such as gamma-interferon.⁽¹⁸⁾ We have hypothesized that mercury may be altering the balance between the production of TH1 and TH2 cytokines in humans. In the present study, we have tested this hypothesis by examining the effect of mercury on the *in vitro* production by human PBMC of a TH2 cytokine (IL-4) and a TH1 cytokine (gamma-IFN).

MATERIALS AND METHODS

Study subjects: After informed consent, blood was collected from healthy volunteers. *Blood collection and cell culture:* 10 ml of blood was collected in

10 ml tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ). For the experiments in which cytokine production between PMA/ionomycin and ConA-stimulated cells in the presence or absence of HgCl_2 was tested (Figure 2), samples from 7 individuals were obtained. For the experiments in which inorganic and organic mercury compounds were compared in regard of cytokine production (Figure 3), 9 samples were obtained from 8 individuals (one individual donated blood on 2 occasions).

Blood was processed immediately. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-hypaque density gradient separation (Pharmacia biotech, AB). The blood sample of each subject was treated as follows: The PBMC layer was harvested and cells were resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 with L-glutamine (Cellgro, Mediatech Inc., Herndon, VA), FCS (10%), penicillin G (100 U/ml) and streptomycin (100 ug/ml) (Gibco, Invitrogen Corporation, Carlsbad, CA) and 1 ml of the cell suspension was given in each well of a 12-well culture plates (1 Mio cells/ml/well). As 1 well was left unstimulated, different stimulants were added to the rest of the wells: phorbol myristic acetate (PMA) 50 ng/ml (Sigma-Aldrich, St. Louis, MO) plus ionomycin 500 ng/ml (Sigma-Aldrich, St. Louis, MO) or with concanavalin A (Con A) 2.5 ug/ml (Amersham Pharmacia biotech, Piscataway, NJ).

To some of these wells, CH_3Hg or HgCl_2 were added in different concentrations ($\text{CH}_3\text{Hg OH}$ 0.1–10 uM and HgCl_2 10–70uM, both solved in PBS). The cells were then incubated at 37°C, in CO_2 5% and 95% humidity for 4 days. Culture supernatants were collected on day 4 and supernatants were kept frozen at -20° until used for further analysis.

Viability testing was attempted by using trypan blue staining and flow cytometric assays with annexin and 7-AAD staining. However, cell viability could only be determined on day 1 because of extensive cell cluster formation on the following days. The viability in all cultures was >80% at day 1 and were estimated to be <50% on day 4 in mercury containing cultures by trypan blue staining (data not shown).

IL-4 and gamma-IFN detection by ELISA: For human IL-4, a Quantikine high sensitivity ELISA kit developing systems was used for low concentrations of IL-4 (sensitivity range 0.5 pg/ml to 15 pg/ml; R&D systems, Minneapolis, MN). For IL-4 concentrations >15 pg/ml., we used a Duo Set ELISA kit developing system (sensitivity range: 15 pg/ml to 1000 pg/ml; R&D systems, Minneapolis, MN). For human gamma-IFN, a Duo Set ELISA kit developing system (sensitivity range: 15 pg/ml to 1000 pg/ml; R&D systems, Minneapolis, MN) was used according to the manufacturer's protocol.

Statistical analysis: Statistical analysis was done by using the InStat program (Graphpad). The arithmetic mean and standard deviations of the cytokine

levels in each group of samples were calculated. Statistical significance between cytokine levels of sample groups was calculated using the Wilcoxon matched-pairs signed rank test. The study was approved by the Institutional Review Board of the Albert Einstein College of Medicine/Montefiore Medical Center.

RESULTS

Comparative Effects of Mercury on PMA/Ionomycin and Con A-Induced Cytokine Production

Initial experiments were performed with inorganic mercury in order to determine its effects on cytokine production. Figure 1 summarizes the main experimental steps.

In the first series of experiments, we tested 2 different cell stimuli (PMA/ionomycin and Con A) to see if the observed effects of mercury were dependent on the method of cell stimulation. Blood samples were obtained from 7 healthy individuals and PBMC were separated and suspended in culture medium. The cultures were then stimulated with either PMA/ionomycin or Con A in the presence or absence of HgCl_2 . We chose to use a concentration of HgCl_2 35 μM for this experiment because preliminary experiments indicated that concentrations between 20 and 50 μM of HgCl_2 induced the highest production of IL-4 in stimulated PBMC (data not shown). PMA-stimulated cells produced very little IL-4 (1.5 $\pm$ 1 pg/ml) (Figure 2A). When HgCl_2 was added to these

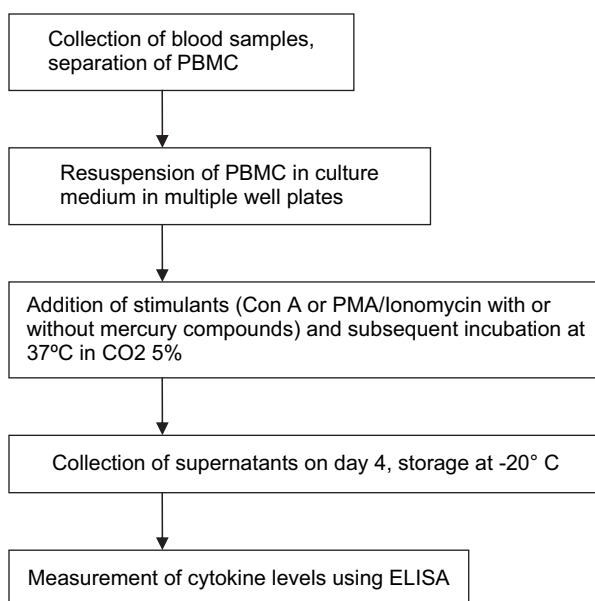


Figure 1: Main experimental steps.

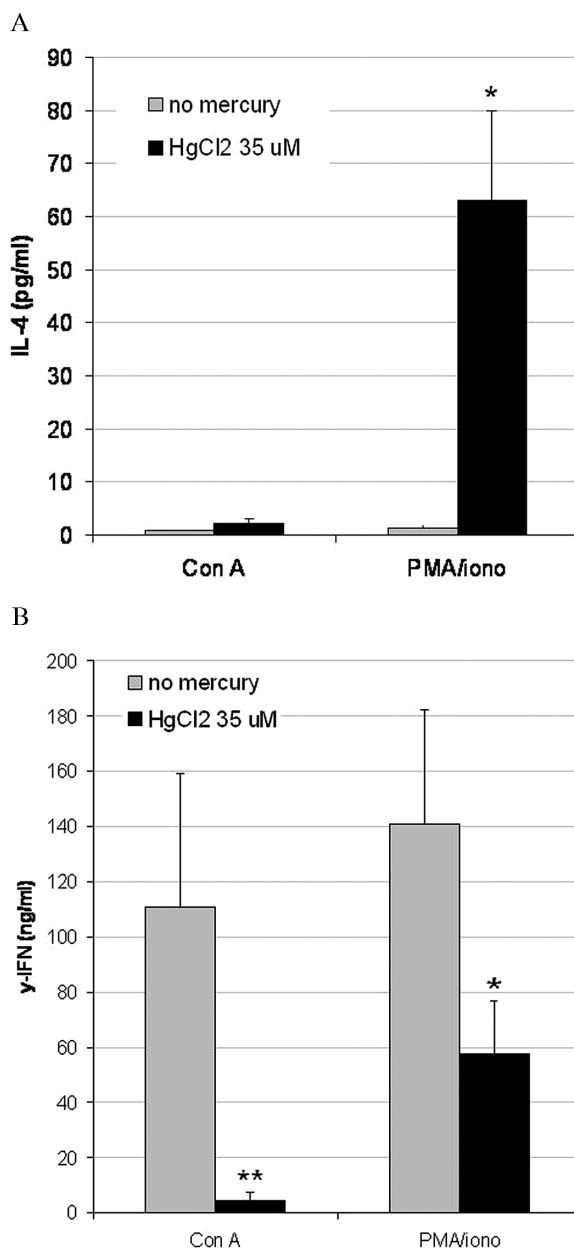


Figure 2: Effect of mercury on PMA/ionomycin or Con A-induced IL-4 production (A) and gamma-IFN production (B). Results represent the arithmetic mean + S.E.M. of 7 samples. Statistical significance was determined by using the Wilcoxon matched pairs signed rank test. A. * $p = 0.02$. B. ** $p = 0.01$, * $p = 0.03$.

PMA/ionomycin stimulated cells, IL-4 production increased significantly (to 63 ± 46 pg/ml, $p = 0.02$). The effect of mercury on Con A-stimulated cells was much less striking. IL-4 production in Con A stimulated PBMC was 0.8 ± 0.77 pg/ml

in the absence of HgCl_2 and only increased to 2.1 ± 2.3 pg/ml when HgCl_2 was added ($p = 0.31$).

At the same concentration, mercury markedly suppressed both Con A and PMA-stimulated gamma-IFN production. In general, gamma-interferon production by PBMC was about 1000 times greater than IL-4 production (ng/ml vs. pg/ml). Both Con A and PMA/ionomycin stimulated PBMC to produce large amounts of gamma interferon (Figure 2B). When HgCl_2 was added to Con A stimulated cells, gamma interferon production dropped from 111 ± 136 pg/ml to 4.4 ± 4.4 pg/ml ($p = 0.01$). The effect of mercury on PMA-stimulated gamma interferon production was somewhat less than its effect on Con A stimulated gamma interferon. PMA/ionomycin stimulated cells produced 141 ± 115 pg/ml of gamma interferon. This level dropped to 58 ± 53 pg/ml ($p = 0.03$) in the presence of mercury.

Comparative Effects of Organic Methyl Mercury (CH_3Hg) and Inorganic Mercury (HgCl_2) on PMA/Ionomycin-Stimulated IL-4 Production

In the next series of experiments, we compared the effects of inorganic mercury to that of methyl mercury, since the latter compound is the major form of mercury to which humans are exposed. We focused on PMA/ionomycin as a stimulant because it was more potent than Con A as a stimulator of IL-4 in response to mercury, as shown in Figure 2. 9 PBMC samples were incubated with either PMA/ionomycin alone or PMA/ionomycin plus CH_3Hg or HgCl_2 at different concentrations. Unstimulated PBMC produced very little IL-4 (2 ± 5 pg/ml, data not shown). Addition of PMA/ionomycin induced a significant increase in IL-4 production compared to unstimulated cells (22 ± 23 pg/ml, $p = 0.004$, Figure 4). When CH_3Hg was added to PMA/ionomycin stimulated cells in increasing concentrations, there was an observable increase in IL-4 production at CH_3Hg 0.1 uM (35 ± 36 pg/ml) compared to PMA-stimulated cells that were cultured in absence of mercury ($p = 0.06$) (Figure 3A). IL-4 production peaked at 2 uM concentrations of CH_3Hg (70 ± 84 pg/ml, $p = 0.03$) and then declined at higher concentrations. In contrast, HgCl_2 induced IL-4 production only at much higher doses. Increased IL-4 production began to occur at HgCl_2 levels of 10 uM and peaked at 20 uM ($p = 0.01$). Preliminary experiments had shown that HgCl_2 levels lower than 10 uM did not increase IL-4 levels (data not shown). IL-4 production began to drop at 50 uM concentrations of HgCl_2 .

Comparative Effects of Organic Methyl Mercury (CH_3Hg) and Inorganic Mercury (HgCl_2) on PMA/Ionomycin-Stimulated Gamma Interferon Production

The supernatants obtained in the experiments shown in Figure 3A were also analyzed for gamma-IFN production (Figure 3B). Unstimulated PBMC

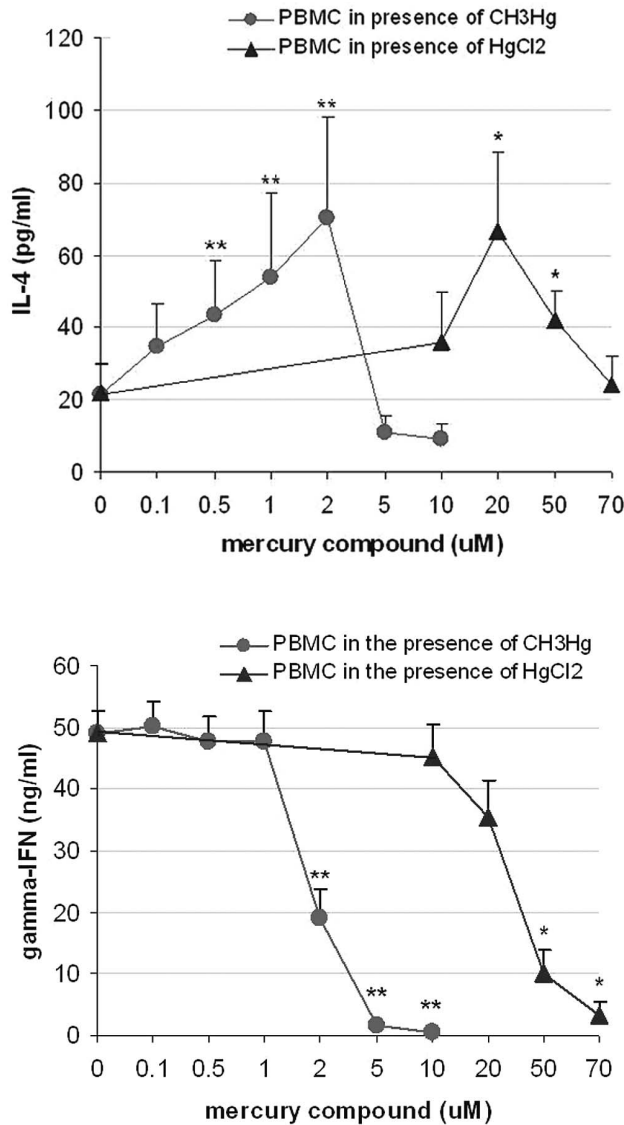


Figure 3: Comparative effects of organic methyl mercury (CH₃Hg) and inorganic mercury (HgCl₂) on PMA/ionomycin-stimulated IL-4 production (A) and gamma-IFN production (B) by human PBMC *in vitro*. Results represent the arithmetic means $\pm$ the S.E.M. of 9 samples. Statistical significance was determined by using the Wilcoxon matched pairs signed rank test comparing the cytokine levels in presence of the mercury compounds with cytokine levels at baseline (in absence of mercury). A. ** $p < 0.01$, * $p < 0.05$. B. ** $p = 0.004$, * $p < 0.01$.

produced some gamma-IFN (0.3 ± 0.8 ng/ml, data not shown) and addition of PMA/ionomycin produced a significant increase in gamma-IFN production (49 ± 10 ng/ml, $p = 0.004$). When PMA-stimulated cells were incubated in the presence of CH₃Hg, gamma-IFN production started to drop at concentrations

of 2 uM (19 ± 14 ng/ml, $p = 0.004$) and was almost totally abolished at CH₃Hg concentrations of 5 uM. As was observed with IL-4, much higher concentrations of HgCl₂ were required to suppress gamma-IFN production. Gamma-IFN production only started to decrease at concentrations of HgCl₂ of 20 uM (35 ± 18 ng/ml, $p = 0.07$) and was significantly decreased at HgCl₂ levels of 50 uM (10 ± 11 ng/ml, $p = 0.004$) compared to baseline.

DISCUSSION

These experiments demonstrate that both inorganic mercury (HgCl₂) and organic mercury (methyl mercury, CH₃Hg) are capable of increasing IL-4 production in stimulated human PBMC *in vitro*. IL-4 production occurred in a dose-dependent fashion although methyl mercury was found to be much more potent than inorganic mercury in inducing IL-4 production. CH₃Hg induced IL-4 production was observed at concentrations starting at 0.1–0.5 uM. In addition, gamma-IFN production was suppressed at slightly higher concentrations of CH₃Hg (2 uM). In contrast, HgCl₂ induced IL-4 production and suppressed gamma-IFN production only at much higher concentrations (20 uM). This increased immunological activity of methyl mercury is consistent with the findings of Shenker et al. who found that lymphocyte apoptosis is induced by methyl mercury at concentrations 5–10 times higher than mercuric chloride.⁽¹⁹⁾ These findings demonstrate that methyl mercury is capable of inducing TH2 cytokine production in humans and alteration of the Th1/Th2 cytokine balance by simultaneous suppression of gamma-IFN.

Although intriguing, there are a number of unanswered questions related to these findings. One is the biological relevance of these *in vitro* phenomena. In this regard, the first issue is whether the concentrations of mercury that are active *in vitro* are comparable to those found in human lymphoid tissues. It is known that mercury accumulates in human and animal nerve tissue and renal parenchyma, but there is less data about mercury accumulation in lymphoid organs. The only published mercury measurements that we could find come from a study that examined tissue mercury levels from 2 infants (ages 2 and 3) that had died from sudden infant death syndrome.⁽²⁰⁾ Their spleen mercury levels were 0.06 and 0.16 ppm, which represents approximately 0.3 and 0.8 uM concentrations of mercury.

Interestingly these spleen levels correspond well with hair mercury levels in children in the same age group while blood mercury levels are about 160 times lower and seem to poorly reflect accumulated mercury.^(21,22) Additional evidence that mercury accumulates in spleen comes from animal studies. Twelve weeks after inserting amalgam dental restorations in rats, spleen mercury levels were about 40-fold higher than blood levels.⁽⁵⁾ Our *in vitro* experiments showed that methyl mercury is capable of inducing IL-4 production starting at 0.1 uM (approximately 0.02 ppm) and peaked at 1–2 uM

(approximately 0.2–0.4 ppm). These findings suggest that methyl mercury may be immunologically active at levels that are present in human lymphoid organs.

A second question related to the issue of potential biological relevance, is whether increased levels of mercury in humans are epidemiologically associated with immunological alterations. Recently, an association between increased urine mercury levels and activity of atopic eczema in young children (beginning school) was reported.⁽¹⁴⁾ Uncovering additional associations may prove difficult because of confounding factors (e.g., certain studied populations that are exposed to high levels of methyl mercury by fish consumption might also have a lifestyle that is not conducive to the development of allergic disease). In addition, genetic variation in the mercury-induced increase in serum IgE and IL-4 expression exists in experimental animals^(8–10) and there is indication for genetic susceptibility to mercury toxicity in human nerve cells.⁽²³⁾

The exact mechanism by which mercury increases IL-4 production is also not yet known. Our findings indicate that mercury significantly augmented IL-4 production induced by PMA/ionomycin but had relatively little effect on IL-4 production induced by the T cell mitogen, Con A. We also tried to stimulate cells with PHA and mercury but no significant increase in IL-4 production could be found (data not shown). PMA exerts its effects on the cell through protein kinase C (PKC).⁽²⁴⁾ PKC plays a critical role in IL-4 production when T-cells are stimulated through their CD3/CD28 receptors.⁽²⁵⁾ Previous studies demonstrated that HgCl₂ (15–20 uM) induced IL-4 gene expression and PKC-dependent calcium influx in mouse T-cell hybridomas. Moreover, HgCl₂ is able to activate PKC directly at higher concentrations (25–100 uM).⁽²⁶⁾

These findings suggest that mercury may enhance IL-4 production in human cells by its effects on PKC activation. Mercury also suppresses gamma interferon production and it is possible that this contributes to the increased IL-4 production, since gamma-IFN is known to inhibit IL-4 production. The decrease of gamma-IFN production could be due to a direct toxic effect on T cells, the presumptive source of gamma interferon in the PBMC that we studied. This interpretation is supported by the findings of Shenker et al. who found that T-lymphocytes exhibit signs of apoptosis starting at 1–2 uM in response to methyl mercury.^(19,27)

It is also possible that the differential effects that we observed might be due to the actions of mercury on two distinct cell types, since PBMC contain also basophils.⁽²⁸⁾ Mercury could be promoting IL-4 production by basophils rather than by T lymphocytes, since there is some evidence that basophils are the major source of IL-4 production in human peripheral blood cells and murine lung.^(29, 30) This would be consistent with our finding that mercury had relatively little effect on IL-4 production by the T cell mitogen, Con A. In contrast, the suppression of gamma interferon production by mercury could be primarily due to its effects on T cells, since Con A-induced gamma interferon

production was affected more by mercury than was PMA-induced gamma interferon production.

In summary, our results support the hypothesis that mercury has the potential to shift the TH1/TH2 balance towards TH2 cytokine production in humans. Whether this phenomenon has clinical relevance at the levels of mercury currently found in most human populations remains to be determined.

ABBREVIATIONS

CH ₃ Hg	methyl mercury
Con A	concanavalin A
Gamma-IFN	gamma-Interferon
HgCl ₂	mercuric chloride
IL-4	interleukin 4
PBMC	peripheral blood mononuclear cells
PMA	phorbol myristic acetate
TH1/TH2	T-helper 1/T-helper 2

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