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Effects of methyl-, phenyl-, ethylmercury and mercurychlorid on immune cells of harbor seals (*Phoca vitulina*)

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Abstract

Mercury (Hg) is present in the marine environment as a natural metal often enhanced through human activities. Depending on its chemical form, Hg can cause a wide range of immunotoxic effects. In this study, the influence of methyl-, ethyl- and phenylmercury as well as mercurychloride on immune functions was evaluated. Two parameters of cellular immunity, proliferation and mRNA cytokine expression of interleukin-2, -4, and transforming growth factor β, were investigated in harbor seal lymphocytes after *in vitro* exposure to Hg compounds. While all Hg compounds had a suppressive effect on proliferation, differences between juvenile and adult seals were found. Lymphocytes from juveniles showed a higher susceptibility to the toxic effect compared to lymphocytes from adults. Furthermore, the degree of inhibition of proliferation varied among the four Hg compounds. The organic compounds seem to be more immunotoxic than the inorganic compound. Finally, for the cytokine expression of methylmercury-incubated lymphocytes, time-dependent changes were observed, but no dose-dependency was found. Marine mammals of the North Sea are burdened with Hg, and lymphocytes of harbor seals may be functionally impaired by this metal. The present *in vitro* study provides baseline information for future studies on the immunotoxic effects of Hg on cellular immunity of harbor seals.

Key words: harbor seal; mercury; lymphocyte proliferation; cytokine expression; North Sea
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Introduction

In the past the North Sea ecosystem was polluted with mercury (Hg), but current studies revealed a trend of reduced input of Hg into the ecosystem (Schmolke *et al*., 2005; Wängberg *et al*., 2007). However, for the German Bight the concentrations of selected metals, including Hg, in water and sediment are still elevated compared to the "Background Reference Concentrations" which the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) derived for the "Greater North Sea" (Schmolke *et al*., 2005). Furthermore, Hg contamination remains a problem for marine organisms due to bioaccumulation in marine biota and biomagnification through the food web. In the Southern and Eastern North Sea, several recent studies measured Hg concentrations in various species including mussel and fish (Baeyens *et al*., 2003; Bakker *et al*., 2005; Schmolke *et al*., 2005; Kakuschke and Prange, 2007).

As top predators marine mammals take up Hg by consumption of contaminated fish. In the Southern and Eastern North Sea, for example, high concentrations of Hg were found in livers of harbor porpoises *Phocoena phocoena* (Siebert *et al*., 1999; Das *et al*., 2004; Strand *et al*., 2005; Lahaye *et al*., 2007) and harbor seals *Phoca vitulina* (Reijnders, 1980; Skaare *et al*., 1990; Wenzel *et al*., 1993), the main species of mammals in the Wadden Sea as well. A recent study on seals of the Wadden Sea describes Hg concentrations in blood samples between 0.043 and 0.611 μg/mL (Das *et al*., 2008). It is well known that Hg can cause a wide range of adverse health effects in mammalian species (Rawson *et al*., 1993; Siebert *et al*., 1999; Bennett *et al*., 2001).

Various chemical forms of Hg can react differently. Organic Hg seems to be more toxic than inorganic mercury ($HgCl_2$) for animals of higher trophic level (Ullrich *et al*., 2001). Most studies have focused on methyl (Me)-Hg, while organic compounds like ethyl (Et)- and phenyl (Ph)-Hg have been largely ignored. All three forms are produced as industrial compounds, primarily as biocides (Goldmann and Shannon, 2001). Some studies showed the presence of Et- and Ph-Hg in the environment (Hintelmann *et al*., 1995; Cai *et al*., 1997). An input into the marine ecosystem was shown on the Danish west coast near a chemical factory producing fungicides; this caused higher Me- and Ph-Hg concentrations in mussels (Riisgard *et al*., 1985).

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The environmental Hg exposure results in concentrations bioavailable for immune cells and high enough to affect their function. An imbalance of the immune system caused by pollutants may play a significant role in the incidence of infectious diseases in marine mammals. Therefore, studies on grey seals (*Halichoerus grypus*), beluga whales (*Delphinapterus leucas*), and bottlenose dolphins (*Tursiops truncatus*) using *in vitro* tests to investigate the influence of Me-Hg on immune cells have been performed (Betti and Nigro, 1996; De Guise *et al*., 1996; Lalancette *et al*., 2003; Kakuschke *et al*., 2006). However, limited information is available for harbor seals. In our own studies on harbor seals considering metal-specific hypersensitivities, several metals including different mercury compounds were tested (Kakuschke *et al*., 2005, 2008a, 2008b).

In recent years, the effects of different Me-Hg concentrations on lymphocyte proliferation and protein synthesis in harbor seal peripheral blood mononuclear cells (PBMCs), as well as cytokine mRNA expression after a long-term Me-Hg exposure of 72 h were evaluated (Das *et al*., 2008). The present study provides further information on these research topics by evaluating the effects of different Hg compounds on lymphocyte proliferation as well as a dose-dependent cytokine mRNA expression after short-term Me-Hg exposure of 6, 24, and 48 h.

1 Materials and methods

1.1 Lymphocyte transformation tests

Metal-specific lymphocyte transformation tests (LTTs) according to the MELISA® (memory lymphocyte immuno-stimulation assay) modification were performed previously for several groups of harbor seals of the North Sea (Kakuschke *et al*., 2005, 2008a, 2008b). For this study, the mercury-specific results of these animals were considered in respect to the different mercury compounds including Me-Hg, Ph-Hg, Et-Hg and $HgCl_2$. Data from 21 harbor seals caught along the Wadden Sea or from animals of the Seal Center Friedrichskoog, Germany, were used. The animals were grouped into juveniles (n = 9, between 1–4 month old) and adults (n = 12, older than one year).

Blood was collected into monovettes with CPDA-solution (Citrate-Phosphate-Dextrose-Adenin) (Sarstedt AG & Co., Nümbrecht, Germany) and stored at room temperature until further processed (not longer than 15 h). The blood was diluted 1:1 with phosphate buffered saline (PBS). Lymphocytes were separated on a Ficoll-Histopaque gradient (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and washed twice in PBS. Cells were resuspended in 20% Medium (RPMI-1640 containing Hepes (Life Technologies GmbH, Karlsruhe, Germany) supplemented with 6.25 mmol/L L-glutamine (Biochrom AG Seromed, Berlin, Germany), 8 mg/L Gentamycin (Sigma-Aldrich Chemie GmbH) and 20% fetal calf serum) and incubated in tissue culture flasks for 30–45 min at 37°C in 5% CO_2 for depletion of monocytes. The lymphocytes were counted and resuspended in 10% Medium to a concentration of 1×10^6 lymphocytes/mL. One milliliter of cell suspension was pipetted into the wells of a 24-well plate (CM-Lab, Vordingborg, Denmark). The cells were cultured for 5 d at 37°C in 5% CO_2 with or without Et-Hg 2-(C_2H_5HgS)$C_6H_4CO_2Na$, Me-Hg ((CH_3)-HgCl), Ph-Hg ($CH_3COOHg(C_6H_5)$) and $HgCl_2$ at concentration of 0.25 and 0.5 μg/mL. The number of cell culture experiments to each Hg compound varied due to different blood sample quantities and numbers of isolated cells. After incubation 600 μL of each cell suspensions were transferred to a new plate and incubated for a further 4 hours with 3°C radioactive marked methyl-^{3}H-thymidine (Amersham Buchler GmbH & Co., KG, Braunschweig, Germany). The cells were then harvested onto filters and the radioactivity measured in a scintillation counter (1450 Microbeta Trilux Wallac Distribution GmbH, Freiburg, Germany).

Incorporation of methyl-^{3}H-thymidine (counts per minute) in the Hg-incubated wells compared to non-Hg-incubated control cultures defined the stimulation index (SI):

$$SI = \frac{\text{Proliferation of Hg – incubated cells}}{\text{Proliferation of non – Hg – incubated cells}}$$

where, SI = 1 reflected no influence of Hg compounds on lymphocyte proliferation compared to non-Hg-incubated (control) cells. SI < 0.1 was regarded as a strong immunosuppressive or cytotoxic influence. SI > 2 was interpreted as a metal-specific hypersensitivity reaction as described for seals (Kakuschke *et al*., 2005, 2006, 2008a, 2008b). Animals showing a Hg-specific hypersensitivity with a SI > 2 were not included in this study.

The Mann-Whitney U Test was used for investigating age and Hg compound correlated differences. Statistical significance was designated as $P \leqslant 0.05$.

1.2 Measurement of cytokine expression using RT-PCR

Primers for the detection of cytokines interleukin (IL)-2, IL-4, and transforming growth factor β (TGF β) were published sequences (Fonfara *et al*., 2008; NCBI GenBank database www.ncbi.nlm.nih.gov) and are shown in Table 1.

In this preliminary study, the cytokine expression of only Me-Hg-incubated lymphocytes from two adult seals (seal I and II) of the Seal Center Friedrichskoog was analyzed. Lymphocytes were separated on a Ficoll-Histopaque gradient and incubated in 96-well plates at 37°C. Each well contained 200000 cells in 100 μL MEME Eagle's Minimum Essential Medium with 10% fetal calf serum. Cells were incubated with 0 μg/mL, 0.05 μg/mL (0.2 μmol/L), 0.1 μg/mL (0.4 μmol/L), 0.5 μg/mL (2 μmol/L) Me-Hg. The total RNA isolation was performed after 6, 24, and 48 h using the Ambion Blood Kit (Ambion Europe, Huntingdon, UK) according to the manufacturers' protocol. RNA was reverse transcribed with murine reverse transcriptase (RT-PCR Core Kit, Applied Biosystems, Weiterstadt, Germany), and the resulting cDNA served as a template for real-time PCR using the Thermocycler MX4000™ (Stratagene Europe, Amsterdam, the Netherlands). The RT-PCR started with an initial step at 95°C for 10 min,