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**Effect of thimerosal on the murine immune system
- especially induction of systemic autoimmunity**

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To Vian and Artin

*And also in loving memory of
Badieh, Ahmad, Jalil, Homayon and Zahida*

ABSTRACT

The organic mercury compound ethylmercurithiosalicylate (thimerosal), an antiseptic and a preservative, has recently raised public health concern due to its presence in vaccines globally. Thimerosal dissociates in the body to thiosalicylate and ethyl mercury (EtHg), which is partly converted to inorganic mercuric mercury (Hg^{2+}). The immunosuppressive, immunostimulatory, and de novo autoimmunogen effect of thimerosal in mice, as well as the accelerating/aggravating effect on spontaneous systemic autoimmunity including dose-response aspects were the subject of this thesis.

Thimerosal perorally (590 μg Hg/kg body weight (bw)/day) to genetically susceptible (H-2^b) mice caused immunosuppression during the first week with reduction of the total number of splenocytes, T- and B-cells. The suppression lasted 2 weeks for CD4⁺ cells, but was superseded by a strong immunostimulation/proliferation including T- as well as B-cells, and polyclonal B-cell activation (PBA). Antinuclear antibodies targeting the 34-kDa nucleolar protein fibrillarin (AFA) appeared after 10 days, followed by renal mesangial and systemic vessel wall immune-complex (IC) deposits. The Lowest Observed Adverse Effect Level (LOAEL) was in the order AFA = glomerular and splenic vessel wall deposits < hyperimmunoglobulinemia < PBA. The LOAEL for AFA was 118 μg Hg/kg bw/day. The LOAEL for the different parameters of this thimerosal-induced systemic autoimmune condition (HgIA) was 3-11-fold higher compared with HgIA induced by HgCl_2 . The thimerosal-induced HgIA shared with HgCl_2 a significant dose-response relationship, and requirement for: T-cells, the costimulatory factor CD28, the IFN- γ /IFN- γ -receptor pathway, but not IL-4. The mRNA expression in lymph nodes of IL-2, IFN- γ , IL-4, and IL-15 was significantly increased but not delayed compared with HgCl_2 .

Treatment with the ubiquitous organic Hg compound methyl Hg using equimolar doses of Hg (533 μg Hg/kg bw/day) caused a transient immunosuppression, followed by a weak immunostimulation and AFA. The IgG AFA isotypes induced by the organic Hg compounds MeHg and EtHg were stable and dominated by a Th1-like pattern over a broad time- and dose range. Treatment with inorganic HgCl_2 caused a dose- and time-dependent pattern of IgG AFA isotypes. Low doses favored a Th1-like pattern, a high dose a balanced or Th2-like pattern. Middle-range doses showed initially a Th1-like pattern which gradually evolved into a balanced or Th2-like pattern. The qualitative difference in IgG AFA isotypes between organic and inorganic Hg may be due to differences in activation and/or suppression of T-helper cell subsets or factors influencing the Th1/Th2-function. Speciation of the renal Hg^{2+} concentration and comparison with the threshold dose for induction of AFA by HgCl_2 showed that even with the lowest doses of thimerosal and MeHg used in this thesis, the AFA response might from a dose threshold point of view have been caused by conversion of the organic Hg species to Hg^{2+} .

Primary treatment with inorganic Hg (HgCl_2) accelerates/aggravates murine systemic autoimmunity, both spontaneous (genetic) and induced by other means. This capacity was assessed for thimerosal over a broad dose range using the (NZB X NZW)F1 hybrid mouse model. Significantly increased antinuclear antibodies (ANA) was seen after 4-7 weeks treatment (LOAEL 147 μg Hg/kg bw/day), and the response was dose-dependent up to 13 weeks. Renal mesangial and systemic vessel walls deposits similar to those in de novo HgIA were present after 7 weeks treatment. Twenty-two to 25 weeks treatment with thimerosal caused, in a dose-dependent fashion (LOAEL 295 μg Hg/kg bw/day), relocation of the spontaneously developing glomerular IC deposits from the capillary vessel walls to the mesangium, which attenuated histological kidney damage and proteinuria, and increased

survival. Thimerosal caused systemic vessel wall IC-deposits over a broad dose range: the No Observed Adverse Effect Level (LOAEL) for renal and splenic vessel wall IC deposits was 18 and 9 $\mu\text{g Hg/kg bw/day}$, respectively. The No Observed Adverse Effect Level could not be determined for the latter, since deposits were present even with the lowest dose used. Thimerosal causes in genetically susceptible mice an initial, transient immunosuppression which is superseded by a strong immunostimulation and systemic autoimmunity, sharing many characteristics with the HgIA induced by inorganic HgCl_2 . The IgG AFA isotype pattern is however qualitatively different, and the threshold dose substantially higher. In contrast, long-term treatment with thimerosal induces systemic vessel wall IC-deposits also using doses below those needed to induce HgIA de novo in H-2^s mice.

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LIST OF ORIGINAL PAPERS

- I** Said Havarinasab, Lars Lambertsson, Johanna Qvarnström, Per Hultman. Dose-response study of thimerosal-induced murine systemic autoimmunity. *Toxicol. Appl. Pharmacol.* 2004; 194:169-179
- II** Said Havarinasab, Bo Häggqvist, Erik Björn, K. Michael Pollard, Per Hultman. Immunosuppressive and autoimmune effects of thimerosal in mice. *Toxicol. Appl. Pharmacol.* 2005; 204:109-121
- III** Said Havarinasab, Erik Björn, Per Hultman. The autoimmunogen effect of the organic mercury species methyl mercury and ethyl mercury. *Manuscript*
- IV** Said Havarinasab, Per Hultman. Alteration of the spontaneous systemic autoimmune disease in (NZB x NZW)F1 mice by treatment with thimerosal (ethyl mercury). *Toxicol. Appl. Pharmacol.*, in press, 2006

The author of this thesis is also a co-author of the following articles:

1. Johanna Qvarnström, Lars Lambertsson, Said Havarinasab, Per Hultman, Wolfgang Frech. Determination of methylmercury, ethylmercury, and inorganic mercury in mouse tissues, following administration of thimerosal, by Species-Specific Isotope Dilution GC-Inductively Coupled Plasma-MS. *Anal. Chem.* 2003; 75; 4120-4124
2. Johan Mellergård, Said Havarinasab, Per Hultman. Short- and long-term effects of T-cell modulating agents in experimental autoimmunity. *Toxicol.*, 2004; 196; 197-209
3. Bo Häggqvist, Said Havarinasab, Erik Björn, Per Hultman. The immunosuppressive effect of methylmercury does not preclude development of autoimmunity in genetically susceptible mice. *Toxicol.* 2005; 206; 149-164
4. Said Havarinasab, Per Hultman. Organic mercury compounds and autoimmunity. *Autoimm. Rev.* 2005; 4; 270-275

ABBREVIATIONS

| | |
|-------------------|---|
| ACA | Antichromatin antibody |
| AFA | Antifibrillar antibody |
| ALP | Alkaline phosphatase |
| AMA | Atomic absorption |
| ANA | Antinuclear antibody |
| ANoA | Antinucleolar antibody |
| APC | Antigen presenting cell |
| BN rat | Brown Norway rat |
| CD | Cluster of differentiation |
| cIg | Cytoplasmic Ig |
| DDTC | Diethyldithiocarbamate |
| DNA | Deoxyribonucleic acid |
| DNP | Dinitrophenyl |
| dsDNA | Double-stranded DNA |
| ELISA | Enzyme-linked immunosorbent assay |
| EtHg | Ethyl mercury |
| FCS | Fetal calf serum |
| FITC | Fluorescein isothiocyanate |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GC-ICP-MS | GC-inductively coupled plasma mass spectrometry |
| GN | Glomerulonephritis |
| GVHD | Graft-versus-host disease |
| HBSS | Hands' balanced salt solution |
| HgCl ₂ | Mercuric chloride |
| HgIA | Mercury-induced autoimmunity |
| HRP | Horse-radish peroxidase |
| IC | Immune complex |
| IF | Immunofluorescence |
| IFN | Interferon |
| Ig(s) | Immunoglobulin(s) |

| | |
|------------|---|
| IIF | Indirect immunofluorescence |
| IL | Interleukin |
| LOAEL | Lowest Observed Adverse Effect Level |
| NOAEL | No Observed Adverse Effect Level |
| MAb | Monoclonal antibody |
| MeHg | Methyl mercury |
| MHC | Major histocompatibility complex |
| OD | Optical density |
| po | perorally |
| PBS | Phosphate-buffered saline |
| PMA | Phorbol myristate acetate |
| RPA | Ribonuclease protection assay |
| ROS | Reactive Oxygen Species |
| sc | Subcutaneous |
| ssDNA | Single-stranded DNA |
| Th | T-helper cell |
| TMAH | Tetramethylammonium hydroxide |
| ZBWF1 mice | (New Zealand Black X New Zealand White)F1 hybrid mice |

INTRODUCTION

Pharmacology and toxicology of mercurials

Mercury and mercurial compounds in general

Mercury is a villain in biological systems with no indications of essentiality for vertebrates. Elemental Hg is however naturally formed in the earth crust through degassing from volcanic areas and oceans [Meili, 1997], and therefore an eternal companion to biological life on earth. Important detoxification mechanisms like glutathione may have evolved to protect organism from high Hg concentrations in a more aggressive atmosphere. In the last century anthropogenic sources of Hg have caused a number of Hg intoxications in individual as well as on group basis.

The three main forms of mercury are: elemental (metallic) mercury (Hg^0), inorganic mercury (Hg^+ and Hg^{2+}), and organic mercury [Goldman and Shannon, 2001]. Elemental Hg emitted from the earth crust and derived from anthropogenic sources may be oxidized to mercuric mercury (Hg^{2+}), and subsequently methylated into methylmercury (MeHg) by a non-enzymatic reaction between Hg^{2+} and a methylcobalamine compound produced by bacteria [Wood, 1983]. This reaction takes place primarily in aquatic systems, and allows MeHg to gain access to the food chain, which is the most important way of Hg exposure in non-amalgam bearers. Organomercurials like MeHg and ethyl Hg (EtHg) contain shorter or longer alkyl or aryl compounds, where Hg (as Hg^+ or Hg^{2+}) is joined with an alkyl- or aryl group by removing one hydrogen atom bound to an alkane, benzene ring or benzene derivative. The type of anion attached to methylmercury affects neither the distribution in the body nor the toxicity [Ulfvarson, 1962; Suzuki, 1973], while the organic radical has a strong impact on both [Magos, 2003]. Today, the most important sources of human exposure to mercury is dental amalgam mainly in the form of elemental mercury, intake of fish (MeHg), and as a preservative thimerosal (thimerosal-EtHg) (reviewed in [Clarkson *et al.*, 2003; Tchounwou *et al.*, 2003]).

Thimerosal

Thimerosal (sodium ethylmercurithiosalicylate) is the sodium salt of a complex formed between thiosalicylic acid and the ethylmercuric ion (see cover), and has during the 20th century frequently been used as an antiseptic and preservative due to its antimicrobial effects, and is still widely used in vaccines globally [Bigham and Copes, 2005] since the 1930's [Pless and Risher, 2000]. It has been reported that thimerosal was used as a preservative in blood plasma by the Britons during W W II in concentration up to 0.1 g/L [Axton, 1972]. Thimerosal contains 49.6% mercury by weight and is dissociated to EtHg and thiosalicylic acid; the thiosalicylic moiety is further decomposed in a multistep procedure [Tan and Parkin, 2000]. The exact steps in the metabolism of the released EtHg is not known [Clarkson, 2002], but includes conversion to mercuric mercury (Hg^{2+}), which at least partly takes place in phagocytic cells of the body [Suda *et al.*, 1993] as well as hepatic microsomes [Suda and Hirayama, 1992]. All Hg ions show a strong binding to thiol (-SH) ligands, which are especially common on glutathione, a tripeptide of cysteine, glutamate and glycine occurring in a high concentration (mM) in the cell [Sanfeliu *et al.*, 2001], and thought to act as a detoxifier of heavy metals including mercurials. However, if glutathione is depleted, free mercury will increasingly bind to the cysteine thiol groups found in many essential cellular proteins, which affects cell functions even at low doses [Wang and Horisberger, 1996], and increases the level of reactive oxygen species (ROS) leading to cell damage and apoptosis in vitro [Macho *et al.*, 1997]. MeHg has recently been claimed to be present in tissues to a large extent as methyl-Hg-cysteine [Harris *et al.*, 2003]. To what extent, if at all, ethyl-Hg-cysteines are present in tissues is unknown. While EtHg has certain similarities to MeHg such as chemistry, initial distribution in the body, and brain damage at sufficient doses [Clarkson, 2002], there are also important differences, not least from a pharmacokinetic point of view. First, EtHg is more rapidly cleared from the body [Magos, 2003; Burbacher *et al.*, 2005], which is manifested as a shorter half-life in blood, ca 50 days for MeHg [Smith and Farris, 1996] and less than 10 days for EtHg [Pichichero *et al.*, 2002; Burbacher *et al.*, 2005]. Secondly, higher levels of EtHg in the kidney and liver, but lower levels in the brain, as compared with MeHg has been shown in short-term studies in mice [Suzuki

et al., 1963] and long-term studies in rats [Ulfvarson, 1962]. Part of this might be due to the lack of an active mechanism for transport of EtHg over the blood-brain barrier as opposed to the large neutral amino acid transporters available for MeHg [Simmons-Willis *et al.*, 2002]. However, once in the brain the proportion of inorganic Hg to the organic compound is several folds higher for EtHg than for MeHg. The exact contribution of organic and inorganic Hg to the brain damage seen after exposure to organic Hg compounds has yet to be determined.

Disease manifestation from exposure to Hg

The toxicity of mercury in both human and animals is dependent on exposure route, frequency, dose, nutritional status, individual susceptibility, and genetic predisposition [Tchounwou *et al.*, 2003]. The knowledge on toxicological effects of mercury on humans stems from poisoning incidents in Japan [Tezuka *et al.*, 1986] and Iraq [Bakir *et al.*, 1973], and from occupational exposure to mercury which have been associated with central nervous system effects [Aschner *et al.*, 1997; ATSDR, 1999; Risher *et al.*, 2002], proteinuria and nephritic syndromes [Kazantzis *et al.*, 1962; Cameron and Trounce, 1965], immunostimulation (reviewed in [Sweet and Zelikoff, 2001]), and immunosuppression (reviewed in [Descotes, 1986]).

Effect of inorganic and organic mercury on the immune system

Immunosuppression

Mercurial compounds have since long been regarded as immunosuppressive substances, which is especially true for the organic compounds.

MeHg is a well-known immunotoxic substance (reviewed in [Descotes, 1986]). In vitro MeHg reduces T- and B-cell responses [Nakatsuru *et al.*, 1985; Brown *et al.*, 1988; Shenker *et al.*, 1992]. In vivo, immunosuppression has been found after exposure to sufficient doses of MeHg. Short-term treatment (up to 1 week) with very high doses (corresponding to 3000-9000 $\mu\text{g Hg/kg bw/day}$) reduces primary and secondary immune response in rodents [Ohi *et al.*, 1976; Hirokawa and Hayashi, 1980;

Brown *et al.*, 1988] and may even cause atrophy of the immune system [Klein *et al.*, 1972; Hirokawa and Hayashi, 1980]. More modest MeHg doses (130-600 µg Hg/kg bw/day) caused in mice after 3 weeks treatment reduced primary and secondary immune responses [Blakley *et al.*, 1980], and after 12 weeks treatment thymic atrophy, reduced NK cell activity [Ilbäck, 1991] and impaired ability to handle viral infections [Koller, 1975; Ilbäck *et al.*, 2000]. A recent study in A.SW mice (H-2^b) using an internal dose of 540 µg Hg/kg bw/day as MeHg caused a transient immunosuppression during the first week of treatment [Haggqvist *et al.*, 2005]. For thimerosal, there are no data on immunosuppression, except that a sufficient dose of thimerosal causes apoptotic cell death in the Jurkat T-cell line [Makani *et al.*, 2002]. For inorganic mercury there are ample evidence of an in vitro immunosuppressive effect on both T- [Shenker *et al.*, 1992] and B-cells [Daum *et al.*, 1993; Shenker *et al.*, 1993]. Murine in vivo studies on the effect of HgCl₂ are less clear, since a range of doses (300-1800 µg Hg/kg bw/day) have shown no significant suppression measured as the number of T- and B-cells [Dieter *et al.*, 1983; Brunet *et al.*, 1993; Johansson *et al.*, 1997; Johansson *et al.*, 1998].

Immunostimulation

Lymphocyte proliferation induced by HgCl₂ occurs in human [Schopf *et al.*, 1967; Caron *et al.*, 1970], guinea pigs, rabbits, rats [Pauly *et al.*, 1969; Nordlind, 1983], and mice [Jiang and Moller, 1995; Pollard and Landberg, 2001]. The reaction is dependent on MHC class II [Hu *et al.*, 1997] and costimulatory molecules, especially IL-1, in mice [Pollard and Landberg, 2001], which in combination with the oligoclonal murine T-cell response in vitro [Jiang and Moller, 1996] as well as in vivo [Heo *et al.*, 1997] makes it possible that the reaction is antigen-dependent although the antigen(s) is (are) presently unknown. Secondary effects of a polyclonal activation of T-cells by mercury are B-cell activation and Ig isotype switching due to cytokines such as IL-4 and IFN-γ. A number of clearly non-antigen specific proliferative effects of Hg have been however been reported in vitro: increase in intracellular calcium [Tan *et al.*, 1993], aggregation of transmembrane CD4, CD3, CD45 and Thy-1 receptors on T-cells with

increased tyrosine kinase p56^{lck} [Nakashima *et al.*, 1994], and attenuation of lymphocyte apoptosis [Whitekus *et al.*, 1999] due to interference with the *Fas-Fas*-ligand interaction in vitro [McCabe *et al.*, 2003]. Exaggerated proliferation and defective apoptosis might cause not only expansion of peripheral lymphocytes, but also allow autoreactive T-cells to escape IFN- γ -dependent activation-induced cell death. This unspecific lymphoproliferative response to Hg is complemented by a Hg-specific proliferation occurring as part of an autoantigen-specific response (see below).

Mercury-induced autoimmunity (HgIA)

Mercury-induced autoimmunity (HgIA) has been described in rabbit [Roman-Franco *et al.*, 1978], rats [Fournie *et al.*, 2001], and in mice [Pollard *et al.*, 2005]. The effect of Hg on the immune system in HgIA can be divided into lymphoproliferation, hypergammaglobulinemia, and autoimmunity manifested as specific autoantibody production and immune-complex disease [Pollard and Hultman, 1997].

Hg-induced autoimmunity in rats

Data on Hg-induced autoimmunity in rats have recently been summarized [Fournie *et al.*, 2001]. Briefly, rat T-cells exhibit upon contact with Hg a stimulation of the early steps in T cell activation, mimicking the effect of T-cell receptor cross-linking, and leading to a polyclonal activation of both T- and B-cells. The frequency of autoreactive anti-MHC class II T cells increases drastically in the susceptible Brown Norway (BN) strain, which also shows a defective IFN- γ -production but enhanced IL-4-production in the CD8 compartment, while the resistant Lewis strain exhibits a reciprocal cytokine pattern. These reactions lead to lymphoproliferation and hyperimmunoglobulinemia (mainly of the Th2 type) in the BN strains, producing anti-basement membrane (anti-laminin) and anti-DNA antibodies. The manifestations are severe with fatalities, but in surviving rats the disease subsides after 4-5 weeks first going through a quiescent state with systemic IC-deposits even if treatment with Hg is pursued. From this point, the rats are resistant to renewed induction of autoimmunity with Hg, an effect mediated via CD8+ cells [Mathieson *et al.*, 1991; Field *et al.*, 2003].

Hg-induced autoimmunity in mice

While there are phenotypic similarities between the autoimmune reaction to Hg in rats and mice, it is now clear that the mouse model of HgIA shows many specific characteristics. The ability of mercury to cause lymphoproliferation in mice is virtually strain-independent since the DBA/2 strain was the only strain out of 22 lacking lymphoproliferation in the popliteal lymph node test, which also included strains with the same MHC-haplotype as DBA/2 [Stiller-Winkler *et al.*, 1988]. These *in vivo* findings show similarities with the *in vitro* effects demonstrated for Hg (see above), and can be assessed by the use of polyclonal B-cell activation markers such as anti-ssDNA and anti-DNP antibodies and serum IgM [Izui *et al.*, 1979]. The other main characteristics in Hg-induced murine autoimmunity, hyperimmunoglobulinemia, is not likely to be due to a direct effect of Hg on B-cells, which are *in vitro* 10-fold more sensitive to the cytotoxic effect of Hg compared with T-cells [Daum *et al.*, 1993]. Instead, an initial polyclonal activation of both T helper type 1 and T helper type 2 cells induces B-cell-stimulating and switching factors such as IFN- γ and IL-4 [Haggqvist and Hultman, 2001], which leads to B-cell proliferation and Ig production [Johansson *et al.*, 1998]. A number of cytokines are increased in murine HgIA induced by inorganic Hg either locally at the site of administration (TNF- α and IL-1 β , IL-6 and IL-10) [Pollard *et al.*, 2005] or systemically (IL-2, IFN- γ , IL-15, IL-4) [Haggqvist and Hultman, 2001], but only IFN- γ has been found to be of paramount importance for the various aspects of the HgIA in mice [Kono *et al.*, 1998; Pollard *et al.*, 2005].

The third characteristic of murine HgIA is induction of autoantibodies to nuclear antigens. While antichromatin abs are seen in certain of the HgIA-susceptible mouse strains [Hultman *et al.*, 1989; Pollard *et al.*, 2005], the main autoantibody response in Hg-treated mice is directed against nucleolar antigens, especially the 34-kDa U3 small nucleolar ribonucleoprotein particle component fibrillar protein [Hultman *et al.*, 1989; Reuter *et al.*, 1989], although it has recently been discovered that other, hitherto unidentified nucleolar antigens may also be the targets in HgIA [Yang *et al.*, 2001]. Intriguing, the molecular specificity of the murine Hg- induced antifibrillar protein antibodies (AFA) is similar to the specificity of AFA in a subset of patients with

systemic sclerosis [Arnett *et al.*, 1996]. The restricted AFA-response in Hg-treated mice is critically dependent on certain MHC class II (H-2) haplotypes (*s*, *q*, *f*, *p*), specifically the I-A locus genes [Robinson *et al.*, 1986; Mirtcheva *et al.*, 1989; Hultman and Eneström, 1992], T-cells [Hultman *et al.*, 1995], costimulatory molecules such as CD40-CD40L and B7-CD28 [Bagenstose *et al.*, 2002; Zheng and Monestier, 2003; Pollard *et al.*, 2005], and IFN- γ [Kono *et al.*, 1998]. The molecular-biochemical events underlying induction of AFA are not known in detail. However, a number of observations have been made. First, Hg interacts directly with fibrillar/fibrillar peptides causing a physically altered molecule [Pollard and Hultman, 1997]. Secondly, Hg-induced cell death modifies the cleavage pattern for fibrillar, resulting in neo-peptides, and exposing cryptic epitopes [Pollard *et al.*, 1997]. Since exposure to Hg with cell death is able to create a 19-kDa immunogenic fragment of fibrillar even without direct molecular interaction between fibrillar and Hg, the new cleavage pattern is likely to be of prime importance [Pollard *et al.*, 2000]. These observations are also in line with the observation that T-cell clones obtained during the first weeks from Hg-treated mice, but not from native mice (H-2^s), proliferate in response to nuclear material complexes with Hg, but only weakly to nuclear material not treated with Hg [Kubicka-Muranyi *et al.*, 1995]. Furthermore, after 8 weeks Hg treatment the response was equally strong to native and Hg-modified material [Kubicka-Muranyi *et al.*, 1996], which may indicate a loss of tolerance by epitope spreading [Vanderlugt *et al.*, 1998].

Finally, the immunopathology of Hg-induced autoimmunity is derived from deposition in the renal glomerular mesangium and systemically in vessel walls of immune-complexes consisting of IgG and complement [Hultman *et al.*, 1996]. The mice exhibit a mild glomerulonephritis with mild proteinuria but not vasculitis or severe signs of tissue damage. AFA have been eluted from kidneys with deposits [Hultman *et al.*, 1989; Robinson *et al.*, 1997]. The lack of tissue IC deposits after treatment with silver [Hultman *et al.*, 1994; Hultman *et al.*, 1995] and Au (Hultman & Pollard, unpublished), indicates that the mere presence of serum AFA is not sufficient to induce IC-deposits, as also shown by [Robinson *et al.*, 1997].

MeHg-induced HgIA has recently been described [Hultman and Hansson-Georgiadis, 1999; Haggqvist *et al.*, 2005]. The main characteristics are a preserved albeit weaker ANoA/AFA response, a weak IL-4 mRNA response, but no significant increase of IL-2 or IFN- γ , no polyclonal B-cell activation, and no systemic tissue IC deposits [Pollard *et al.*, 2005].

Acceleration of spontaneous and induced autoimmune diseases by Hg

Environmental and other agents may induce various phenotypic expressions of autoimmune diseases due to interaction with different genotypes. This includes the possibility not only of de novo autoimmune disease, but also an acceleration and aggravation of autoimmune conditions with other primary genetic or non-genetic etiologies. For example, in experimental models, the polyclonal B-cell activating agent lipopolysaccharide-lipid A portion [Hang *et al.*, 1983], UV radiation [Ansel *et al.*, 1985], halothane [Lewis and Blair, 1982], and polyinosinic-polycytidylic acid [Carpenter *et al.*, 1970] accelerate spontaneous autoimmune disease manifestations.

Recently, inorganic Hg was shown to accelerate the spontaneous autoimmune manifestations in the NZBWF1 mouse strain as evidenced by lymphoid hyperplasia [Pollard *et al.*, 1999], polyclonal B-cell activation [al-Balaghi *et al.*, 1996], hyperimmunoglobulinemia and antichromatin antibodies [Pollard *et al.*, 1999], as well as immune-complex deposits [Abedi-Valugerdi *et al.*, 1997; Pollard *et al.*, 1999]. Hg treatment of the MRL^{+/+} strain and the autoimmune-prone, *Fas*-deficient MRL^{*lpr/lpr*} strain caused severely and slightly accelerated autoimmune manifestations, respectively [Pollard *et al.*, 1999]. However, recent studies have shown that the autoimmunity may be severely aggravated also in the MRL^{*lpr/lpr*} strain, provided that a lower dose of Hg is administered [Pollard *et al.*, 2005]. Using the AKR strain, which is H-2 congenic with the MRL strains, aggravation of the autoimmune disease was linked to non-MHC genes [Pollard *et al.*, 1999]. Studies in the autoimmune-prone BXSB and the non-autoimmune C57BL/6 strains, which share the H-2^{*b*} haplotype, showed that Hg triggers the *Yaa*-gene dependent lupus-like autoimmune disease in

BXSB mice by aggravating lymphoid hyperplasia, antichromatin antibodies, and glomerulonephritis, while Hg had little effect on the MHC-congenic C57BL/6J strain, linking the genetic susceptibility for acceleration of autoimmunity by Hg to non-MHC genes also in this disease model [Pollard and Landberg, 2001]. Interestingly, a short course of Hg to BXSB mice was sufficient to cause a life-long increase in the autoimmune response. Furthermore, a dose of Hg which was comparatively lower than the dose accepted in the occupational setting accelerated the spontaneous autoimmune disease in the BXSB mice [Pollard and Landberg, 2001]. While the above observations may give the impression that a sufficient dose of Hg will always accelerate spontaneous autoimmune diseases, it was recently found [Hultman, 2006] that Hg treatment for more than a year in the spontaneous autoimmune (SWR X SJL)F1 mouse model [Vidal *et al.*, 1994], neither accelerated the onset nor increased the severity of the systemic autoimmune manifestations. The proposed explanation is that the SWR strain possesses non-MHC genes that can suppress Hg-induced exacerbation of autoimmunity. Therefore, the effects of autoimmune inductors such as Hg, Ag, and Au, need to be examined in all available spontaneous models of autoimmune disease, since a specific interaction takes place between the genetic factors (especially non-MHC genes), the spontaneous autoimmune conditions, and the specific metal. Finally, Silbergeld *et al.* recently examined if an autoimmune disease caused by a primarily non-genetic mechanism might also be accelerated by Hg [Via *et al.*, 2003; Silbergeld *et al.*, 2005]. A lupus-like chronic graft-versus-host disease (GVHD) was induced using F1-hybrids of two strains resistant to Hg (C57BL/6 and DBA/2), and DBA/2 donor cells. A 2-week exposure to host and donor mice of low-dose Hg (10 µg Hg/kg bw/day) ending one week before GVHD induction aggravated the lupus-like GVHD condition.

Mercury-induced de novo and/or accelerated autoimmunity in humans

Most autoimmune diseases are linked to genetic factors, which is to be expected also from any de novo autoimmune reactions after exposure to Hg. However, the genotypes causing such reactions are unknown in humans. Further increasing these difficulties is

the possibility that the same environmental agent by interacting with different genotypes may result in a number of different phenotypic disease expressions, including a mere acceleration and aggravation of autoimmune diseases with another primary etiology.

A number of case reports have described systemic autoimmune disease manifestations in individuals with a known and distinct significant exposure to Hg occupationally or otherwise [Röger *et al.*, 1992; Schrallhammer-Benkler *et al.*, 1992]. Furthermore, there are a number of reports on immune-mediated kidney diseases after Hg exposure [Enestrom and Hultman, 1995].

The concept of Th1/Th2

In the late 1980s the Th1/Th2 hypothesis emerged from the observations in mice by Mosmann and coworkers suggesting that CD4⁺ T-cells differ in cytokine expression establishing the concept of two subtypes of T-helper cells termed T-helper 1 (Th1) and T-helper 2 (Th2) [Mosmann *et al.*, 1986; Mosmann and Coffman, 1989]. The concept was subsequently confirmed in human T-cells (reviewed in [Abbas *et al.*, 1996]). Cytokines expressed by Th1 cells include interferon- γ (IFN- γ), interleukin-12 (IL-12) and tumor necrosis factor β (TNF- β), in contrast Th2 cells produce IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. However, the cytokines produced by the two subsets varies in different studies. The Th1 cells are believed to promote cellular immune responses and Th2 cells promote humoral immune response by strong antibody responses, including IgE production [Mosmann and Sad, 1996; Szabo *et al.*, 1997; Kono *et al.*, 1998]. Subsequently the dominance of one or the other of the Th-cell pathways may result in a predominantly cellular or antibody response [Mosmann *et al.*, 1986].

In the first contact with antigen and antigen-presenting cells CD4⁺ T-cells secrete low levels of either IL-12, IFN- γ or IL-4. This pre-activation state has been called Th0, occurs within 48 h, and secretion of the Th1 or Th2 cytokine becomes evident upon re-stimulation [Nakamura *et al.*, 1997]. Factors which influence the differentiation along the Th1 or Th2 pathways include the concentration and physical form of antigen, co-

stimulation of the T-cells, the type of antigen-presenting cells, and the route of antigen entry, and the presence of hormones (reviewed in [Seder and Paul, 1994; Abbas *et al.*, 1996; Rengarajan *et al.*, 2000]). Co-stimulation includes for Th2 activation low levels of IL-4 from mast cells [Wu *et al.*, 2001] and eosinophils [Lacy and Moqbel, 2001] and for Th1, IL-12 produced by neutrophils [Fresno *et al.*, 1997] and dendritic cells [Cella *et al.*, 1996]. IFN- α produced by macrophages [Belardelli, 1995] have been shown to be initiator of both Th1 and Th2 differentiation.

The molecular mechanisms underlying Th1 or Th2 cytokine polarization are unknown but activation of signal transducer and activator of transcription (STAT)4 by IL-2 promotes the production of IFN- γ in Th1 cells, and activation of STAT6 by ligation of IL-4 receptor by IL-4 leads to a Th2 cell differentiation (reviewed in [Rengarajan *et al.*, 2000; Theofilopoulos *et al.*, 2001]).

Systemic autoimmune diseases such as murine mercury-induced autoimmune syndrome [Goldman *et al.*, 1991] and lupus erythematosus [Theofilopoulos and Dixon, 1985] were once suggested to be mediated by Th2 cells as well as the insulin-dependent diabetes mellitus which are induced by Th1 cells [Cameron *et al.*, 1997]. However, the Th1/Th2 concept has recently been questioned since cytokines-producing cells may secrete a mixed pattern of both Th1- and Th2-associated [Theofilopoulos *et al.*, 2001; Dent, 2002]. Therefore, linkage of particular diseases to Th1 or Th2 cells is probably an oversimplification.

Effect of glutathione status on the regulation of Th1/Th2-secreting cytokines

It is well-known that mercuric ions have a very high affinity for thiol-containing bio molecules, such as glutathione (GSH), cysteine (Cys) , homocysteine (Hcy), N-acetylcysteine (NAC), metallothionein (MT) and albumin [Bridges and Zalups, 2005]. GSH is a protective and regulatory antioxidant known to influence the Th1/Th2 cytokine pattern [Peterson *et al.*, 1998]. A number of investigators have shown that depletion of GSH from antigen-presenting cells (APCs) causes decreased Th1 response [Peterson *et al.*, 1998; Murata *et al.*, 2002]. In the early 1990's van der Meide and collaborators showed that the concentration of GSH in the presence of mercury

regulated IFN- γ production in both Hg-susceptible Brown Norway (BN), and Hg-resistant Lewis rats [van der Meide *et al.*, 1993]. They found that decreased cellular GSH levels correlated with reduced number of IFN- γ producing cells [van der Meide *et al.*, 1993]. Treatment with mercury in BN rats favors a Th2-dominated autoimmune syndrome [Gillespie *et al.*, 1995] and subsequently an increased IL-4 expression. Furthermore, up-regulation of IL-4 showed an inverse correlation with intracellular GSH in BN rats treated with HgCl₂ [Wu *et al.*, 2001].

AIMS

General aim

To study de novo induction of mouse systemic autoimmune disease and acceleration/aggravation of spontaneous murine systemic autoimmunity, by the organic mercury species ethyl mercury (in the form of thimerosal) regarding mechanisms and dose requirements.

Specific aims

Paper **I**: to study in genetically susceptible mice the immunostimulatory and autoimmune potential of thimerosal, especially dose-response relationships

Paper **II**: to examine the immunosuppressive and immunostimulatory effects of thimerosal as well as the expression of/requirement for T-cells and for T-cell factors such as cytokines and co-stimulatory molecules in genetically susceptible mice,

Paper **III**: to discern the possible primary autoimmune effect of ethyl mercury (thimerosal) and methyl mercury compared with the secondary autoimmune effect caused by inorganic mercury formed by conversion of organic mercury in the body

Paper **IV**: to study the ability of thimerosal to accelerate and/or aggravate the spontaneous autoimmune disease in ZBWF1 mice over a wide dose- range.

MATERIAL AND METHODS

Mice

Strains

The female A.SW (H-2^s) mice (I, II and III) and the female B10.S (H-2^s) mice (II) were obtained from Taconic M& B (Ry, Denmark). Female SJL mice (H-2^s) heterozygous (*nu/+*) or homozygous (*nu/nu*) for the *nu*-mutation (nude mice) (II) [Hultman *et al.*, 1995] were obtained from National Institute of Health (Bethesda, MD, USA) and bred in the animal facilities of the Faculty of Health Sciences, Linköping. Female A.TL and B10.TL mice (H-2^d) were originally obtained as breeding pairs from Harlan Ltd. (Oxon, UK) and Department of Immunogenetics, University of Tubingen Germany, respectively. Breeding was maintained by sister-brother mating in the animal facilities of the Faculty of Health Sciences, Linköping. B10.S mice homozygous (-/-) for a targeted mutation (knock out, KO) of the genes for IL-4 [Kuhn *et al.*, 1991], IL-6 [Kopf *et al.*, 1994] or CD28 [Shahinian *et al.*, 1993; Kono *et al.*, 1998; Pollard *et al.*, 2003] were a kind gift from the Scripps Research Institute, La Jolla, CA, USA, and maintained by sister-brother mating in the animal facilities of the Faculty of Health Sciences, Linköping. Mice (H-2^s) homozygous for a targeted mutation of the IFN- γ receptor (B10.S-IFN- γ R-/-) [Huang *et al.*, 1993; Pollard *et al.*, 2003] were maintained at the Animal Department of the Scripps Research Institute, La Jolla, CA. The female (NZB X NZW)F1 hybrid mice used in paper IV were purchased from Harlan Scandinavia (Allerod, Denmark).

Housing

Mice kept in animal facilities of the faculty of Health Sciences, Linköping, were housed under 12 h dark- 12 h light cycles, kept in steel-wire cages and given pellets (type R70, Lactamin, Vadstena, Sweden) and tap water ad libitum. The pellets contained 23 ng Hg²⁺/g and 4 ng methylmercury as Hg/g, whereas the EtHg concentration was below the detection limit (<0.1 ng/g) [Qvarnstrom *et al.*, 2003]. The

SJL-^{nu/+} and nu/nu mice used in paper II were housed under specific pathogen-free conditions given sterilized pellet and water.

Treatment with thimerosal, methyl mercury, or mercuric chloride

Peroral treatment

Throughout the studies thimerosal was administered perorally in drinking water ad libitum. Fresh solutions were prepared once weekly by dissolving 0.156, 0.313, 0.625 (IV), 1.25 (I, IV), 2.5, 5 (I, IV), 10 (I, II, III), 20 or 40 (I) mg thimerosal/L in drinking water. HgCl₂ was given as 0.8, 1.5, 3, 8, or 25 (III) mg/L drinking water. MeHg was given perorally to one group of mice by dissolving 8.2 mg MeHg/L (III) drinking water. Controls were given drinking water without any additions.

Subcutaneous injections

MeHg was given to the mice in 0.8, 1, 1.6, 2 or 4 (III) mg/kg bw as sc injection on the dorsum every third days. One group of mice received 1.7 (III) mg HgCl₂/kg bw as sc injection on the dorsum every third day for 14 days.

Blood and tissue sampling

Blood was obtained during specified points of time during the different experiments and at sacrifice (I-IV) for serological examination. A spot sample of urine was obtained after specified points of time for determination of urinary proteins (IV). Pieces of the kidney and spleen (I-IV) were obtained for determination of immune-complex deposits; of the left kidney and part of the mesenterial lymph nodes for speciation of mercury (II); of the mesenterial lymph nodes for RPA (II), and of the spleen for quantitation of lymphocyte subsets and expression of activation markers by flow cytometry (II).

Determination of urinary proteins

Spot samples of urine from ZBWF1 mice (IV) were analyzed for the presence of proteins, especially albumin, using Combur¹⁰Test[®]M strips (Roche Diagnostics GmbH, Mannheim, Germany) assessed photometrically by Urilux[®]S (Roche Diagnostics GmbH). The test detects the ion concentration of the urine. Control-Test M calibration strips were used before the first samples were read at any measurement time. The result was indicated as 0 (< 0.25 g protein/L), 1 (≥ 0.25 g/L), 2 (≥ 0.5 g/L), 3 (≥ 0.75 g/L), 4 (≥ 1.0 g/L), 5 (≥ 1.5 g/L), 6 (≥ 2.5 g/L), and 7 (≥ 5.0 g/L).

Serum antinuclear antibodies assessed by indirect immunofluorescence (I-IV)

For detection of serum antinuclear antibodies (ANA) indirect immunofluorescence was performed as previously described [Hultman and Eneström, 1988] using sera diluted 1:40 - 1:20,480 which were incubated on slides with a monolayer of HEp-2 cells (Binding Site Ltd, Birmingham, UK), followed by goat anti-mouse IgG antibodies (abs) (I-IV) (Sigma, St Louis, Missouri, USA), IgG1, IgG2a, IgG2b and IgG3 (III) abs (Southern Biotechnology Associates, Inc, Birmingham, USA) diluted 1:50. The ANA titer was defined as the highest serum dilution which showed a specific ANA staining. No staining at a serum dilution of 1:40 was considered as a negative result (0). The slides were assessed in a Nikon incident-light fluorescence microscope (Nikon Instech Co. Ltd., Kanagawa, Japan), using coded samples. Sera from young mice which did not stain the cell nucleus or cytoplasm in an ANA test were pooled and used as a negative control.

In order to compare the presence and titer of ANoA of the IgG1 and IgG2a isotype, an IgG2a/IgG1 index was calculated using titer steps. No specific staining using a serum dilution of 1:40 with anti-mouse IgG1 or anti-mouse IgG2a was considered as "0". Specific staining at 1:40 was considered as one titer step, at 1:80 two titer steps etc. By comparing the titer of IgG2a and IgG1 in terms of the number of titer steps, a net value for ANoA of the isotype IgG2a and IgG1 was determined for each serum. This value was called "IgG2a/IgG1 index". A negative value of the index resulted if the IgG1 titer was stronger than the IgG2a titer.

Serum anti double stranded DNA (dsDNA) by IIF

Anti-double strand DNA (anti dsDNA) Abs were detected using the *Crithidia luciliae* kinetoplast staining assay [Sontheimer and Gilliam, 1978]. Slides with *Crithidia luciliae* (Binding Site) were incubated with serum diluted 1:10. Bound anti-dsDNA Abs were detected by FITC-conjugated goat anti-mouse IgG Abs (Sigma) using a Nikon incident-light fluorescence microscope (Nikon) without knowledge of treatment or other results. A staining of the kinetoplast was defined as a positive reaction while staining of basal body or no staining at all was defined as a negative reaction.

Serum antinuclear antibodies assessed by Immunoblotting (II)

The specificity of the antinuclear antibodies in the serum was assessed by immunoblotting as described before [Warfvinge *et al.*, 1995] with minor modifications. Briefly, mouse liver nucleoli were isolated, aliquots of boiled nucleoli were SDS-PAGE separated using a 12.5 % gel, and electrophoretic transfer to 0.45 μ m nitrocellulose membranes (BioRad Lab, Hercules, CA, USA) was performed for 1 h at 0.8 mA/cm² under water cooling (Criterion Blotter, BioRad Lab). Nitrocellulose strips were blocked in a solution of Tris-buffer (TBS)-5 % non-fat dry milk (blotting grade, BioRad)-0.05 % Tween 20 overnight at 4° C before being incubated with sera diluted 200-fold in TBS-Tween. Bound antibody was detected with horseradish peroxidase-(HRP) conjugated goat anti-mouse IgG (Southern Biotechnology) diluted 1:5,000, followed by enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham, Stockholm, Sweden).

Tissue immune deposits (I-IV)

Pieces of the left kidney and the spleen were examined with direct immunofluorescence as described before [Hultman *et al.*, 1995] using FITC-conjugated goat anti-mouse IgG and IgM Abs, (Sigma), and anti-C3c Abs (Organon-Technica, West Chester, PA, USA), and FITC-conjugated goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 Abs (Southern Biotechnology). The titer of IgG, C3c (I-IV),

IgG1, IgG2a, IgG2b and IgG3 (III, IV) deposits in the glomeruli and vessel walls of the kidney and the spleen was determined either by serial dilution of the Abs to 1:5,120, when the titer was defined as the highest dilution of the respective ab which gave a specific staining, or by assessing the strength of the staining using a fixed dilution of the ab with the use of a scale. The scale used was recorded and graded as 0, absent; +1, scattered deposits; +2, moderate amount of deposits; +3, abundant deposits; +4, filled with deposits. All assessments were made without knowledge of the treatment given or other results.

Tissue mercury concentration

Determination of total mercury concentration in the kidney and the mesenterial lymph nodes

For determination of the total tissue Hg concentration, frozen renal tissues were thawed and cut with a scalpel into 5- to 10-mg pieces that were directly analyzed by atomic absorption spectrophotometry in a Leca AMA 254 mercury analyzer [Bourcier and Sharma, 1981].

Speciation of the renal mercury concentration in mesenterial lymph nodes and kidney

For speciation of Hg^{2+} , MeHg and EtHg a previously described model was used [Qvarnstrom *et al.*, 2003]. Briefly, 6–140 mg of the frozen mouse tissue, from controls and treated mice was thawed, and spiked with 30–200 each of the diluted aqueous standards containing 11–580 ng/mL labeled methyl mercury ($\text{CH}_3^{200}\text{Hg}^+$), ethyl mercury ($\text{C}_2\text{H}_5^{199}\text{Hg}^+$) and inorganic mercury ($^{201}\text{Hg}^{2+}$), respectively. Samples were then digested using 2 mL 20% (w/w) of tetramethylammonium hydroxide (TMAH). The dissolved mercury species were extracted at pH 9 with diethyldithiocarbamate (DDTC) into toluene and reacted with butylmagnesium chloride to form butylated derivatives. The derivatized species were separated and detected by gas chromatography-inductively Coupled Plasma-mass spectrometry.

Assessment of cytokine mRNA by ribonuclease protection assay (RPA) (II)

The mesenteric lymph nodes were carefully dissected, removed from the body and homogenized with an electric homogenizer (Omni-tron 17106, Omni International, Waterbury, CT) in 1 ml of Ultraspec™ (Biotech Laboratories, Inc., Houston, TX), followed by a single-step RNA isolation method, performed according to the manufacturers instruction (Biotech Bulletin no. 28, 1993).

The Ribonuclease protection assay (RPA) has been described previously [Haggqvist and Hultman, 2001]. Briefly, the expression of mRNA for IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-15, IFN- γ , and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assessed with multi-probes for the different cytokines using the RPA method [according to the instructions of the manufacturer (Riboquant® Instruction Manual, 6th ed., Pharmingen, San Diego, CA)]. The RPA multi-probes incorporating [α -32] UTP were transcribed from the template set mCK-1 (Pharmingen, San Diego, CA) with an *in vitro* transcription kit (Pharmingen). 18-20 μ g mRNA from each lymph node was hybridized overnight at 56 °C together with an excess of multi-probes, followed by digestion of unprotected probes with RNase A + T1 mixture. The protected probes were separated by electrophoresis and the polyacrylamide sequencing gel was dried for 1 hour at 80 °C.

A phosphor imaging plate was exposed to the dried gels, and the photo-stimulated luminescence was assessed using a BAS-1000 instrument (Fuji Photo Film Co. Ltd., Japan). The gel images displaying bands representing cytokines and the housekeeping gene GAPDH, which was used for normalization, were analyzed and evaluated with Science Lab 97, Image Gauge 3.01 software (Fuji Photo Film Co. Ltd.).

Enzyme-linked immunosorbent assay (ELISA)

Serum antichromatin antibodies (ACA) (I, IV)

Antichromatin antibodies (ACA) were measured using the method of Burlingame and Rubin [Burlingame and Rubin, 1990]. Calf thymus chromatin (180 μ l/well) in distilled water was added to ELISA plates (Nunc, Copenhagen, Denmark) followed by 20 μ l of

10 X PBS. After overnight incubation at 4°C the plates were post-coated with gelatin, serum diluted 1:400 in PBS was added, followed by washing, alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG abs (Caltag Laboratories, Burlingame, CA, USA), washing and addition of substrate. The optical density was read at 405 nm, and background values were subtracted.

Anti-DNP (I, II, IV)

The method used has been described before [Johansson *et al.*, 1997]. Microtiter plates (Nunc) were coated overnight with human serum albumin conjugated with 30-40 moles dinitrophenyl (DNP) per mole albumin (Sigma). Following repeated washes with BSA-PBS, the wells were incubated with sera diluted 1:100, washed, and alkaline phosphatase (ALP)-conjugated rabbit anti-mouse Ig Abs (reacting with IgG, IgM and IgA) (Sigma) was added. After repeated washes with BSA-PBS, substrate was added, and the reaction stopped with 3M NaOH. The optical density was measured at 405 nm, and the background values in wells coated with PBS were subtracted.

Anti-ssDNA (I, II, IV)

The method used has been described before [Johansson *et al.*, 1997]. Microtiter plates were coated overnight with single-stranded DNA (ssDNA), washed with PBS-Tween 20, blocked with BSA-Tween 20-PBS, repeatedly washed first with PBS-Tween and then with PBS. Sera diluted 1:150 in 1% BSA-PBS were incubated in the wells. The plates were washed six times with BSA-Tween-PBS and incubated with ALP-conjugated rabbit anti-mouse Ig Abs (reacting with IgG, IgM and IgA) (Sigma) diluted in BSA-Tween-PBS. After repeated washing, substrate was added, reaction stopped with 3M NaOH, optical density measured at 405 nm, and the background subtracted. A pool of sera from MRL-^{*lpr/lpr*} mice was used as the positive control. Using a monoclonal antibody (MAb) (clone HB2) reacting with double-stranded (dsDNA) (SeraLab), we detected no contamination with dsDNA in the coating (data not shown).

Serum IgM concentration (I)

For analysis of serum IgM [Johansson *et al.*, 1997] microtiter plates were coated with rat anti-mouse IgM (clone LO-MM-3) monoclonal antibody (Technopharm, Paris, France). Following blocking, the wells were incubated with diluted serum, and bound IgM was detected during diluted horseradish peroxidase-(HRP-) conjugated rat anti-mouse IgM MAb (clone LO-MM-3, Technopharm). The optical density in the wells was measured at 450 nm and the IgM concentration in the samples was derived from a standard curve which was constructed by using mouse myeloma protein of the IgM isotype (clone MANDP-5, Technopharm).

Serum IgE concentration (I, II, IV)

Serum IgE was determined as described before [Warfvinge *et al.*, 1995]. Briefly, microtiter plates were coated overnight with rat anti-mouse IgE Abs (Southern Biotechnology), followed by blocking and incubation with diluted serum. Bound IgE was detected by HRP-conjugated goat anti-mouse IgE Abs (Nordic Immunological Lab, Tilburg, Netherlands). The optical density in the wells was measured at 450 nm and the IgE concentration in the samples was derived from a standard curve using mouse myeloma protein of the IgE isotype (Sigma).

Serum IgG1 concentration (I-IV)

Serum IgG1 was determined as previously described [Johansson *et al.*, 1997]. Briefly, microtiter plates (Nunc, Copenhagen, Denmark) were coated overnight with rat anti-mouse IgG1 MAb (Technopharm) followed by washing and blocking. The plates were incubated with diluted serum and bound IgG1 was detected using HRP-conjugated rat anti-mouse IgG1 MAb (Technopharm). After washing and addition of substrate, the optical density in the wells was measured at 450 nm. Wells incubated with PBS instead of serum were used for assessing the background values which were subtracted. Standard curves using mouse myeloma proteins of the IgG1 isotype (Technopharm) were used to obtain the actual concentration.

Serum IgG2a concentration (I-IV)

In paper I, II, and IV analysis of serum IgG2a concentrations was performed as previously described [Johansson *et al.*, 1997]. Briefly, microtiter plates (Nunc) were coated with purified anti-mouse Ig κ -light Chain (Pharmingen). The wells were washed, blocked and then incubated with diluted sera. Bound IgG2a was detected with alkaline phosphatase (ALP)-conjugated anti-mouse IgG2a (Pharmingen). The optical density in the wells was measured at 405 nm and background values were subtracted as above. The IgG2a concentration in the samples was obtained from a standard curve using purified IgG2a (Pharmingen).

The serum IgG2a concentration in paper III was determined using an ELISA Quantitation Kit (Bethyl laboratories. Inc., Montgomery, TX, USA). Microtiter plates (Nunc) were coated at room temperature with goat anti-mouse IgG2a-affinity purified Abs. Plates were washed, blocked, and the wells incubated with serum diluted 1:16,000. Bound IgG2a Abs were detected with goat anti-mouse IgG2a HRP-conjugated Abs followed by washing and addition of substrate. The optical density in the wells was measured at 450 nm, and the background values in the wells were subtracted. A standard curve was obtained using pooled mouse sera (Bethyl) to determine the actual concentration.

Flow cytometry (II)

Monoclonal antibodies and reagents

Monoclonal antibodies (MAb) were purchased from Becton Dickinson (BD) (San Diego, CA, USA) and Oxford Biotechnology Ltd. (Oxon, UK). RPMI-1640, Fetal Calf Serum (FCS), and Hanks' balanced salt solution (HBSS) 10x were from Gibco (Paisley, UK). Rabbit serum was obtained from Dako (Copenhagen, Denmark) and permeabilizing agents (Perm/Wash and Cytotfix/Cytoperm) were obtained as kits from BD.

Spleen preparation

This method has previously been described [Johansson *et al.*, 1997]. Briefly the spleen was aseptically removed, and single cell suspension was prepared in RPMI 1640 by teasing the spleen followed by pipetting the suspension carefully and centrifuging at 300g for 10 minutes. The cell suspension was kept on ice to reduce capping of the receptors on the cell surface. The red blood cells were lysed with a solution of 0.84% NH_4Cl . After counting the mononuclear cells were diluted in 10 ml tubes for additional preparation.

Two color flow cytometry analysis of cell surface markers and cytoplasmic Ig^+ cells

The splenic cell concentration was adjusted to 20×10^6 mononuclear cells/ml and 50 μl (1×10^6 cells) was incubated with 40% rabbit serum for 20 minutes at 4°C to block the Fc receptors. The cells were washed, and the diluted monoclonal antibodies (MAb) were added to the cells as previously described [Johansson *et al.*, 1997]. 10,000 cells were then acquired and saved in list mode using a LSR flow cytometer (BD). Cells from control and thimerosal-treated mice were prepared and then acquired on the same day in the flow cytometer. Analysis was performed using the Cell Quest software (BD). Dead cells (5-10%) were gated using 7-Amino-actinomycin D (ViaProbe, BD). The number of CD3^+ , CD4^+ , and CD19^+ cells and their fraction in the lymphocyte population was determined. Analysis of activation markers on CD3^+ cells included CD69, CD71, CD122, CD25, CD134 (OX40), and on CD19^+ cells CD71. The geometric mean of fluorescence intensity (MFI) was determined for each activation marker in controls and thimerosal-treated mice at each point of time.

To analyze cytoplasmic Ig^+ cells (cIg^+) 1×10^6 cells were incubated with 250 μl of Cytofix/Cytoperm (BD) for 20 minutes at 4°C , followed by two washes with HBSS-2% FCS buffer and then resuspension in 1 ml Perm/Wash (BD). The MAbs were diluted 1:50 in Perm/Wash solution, and incubated with the cell suspensions for 30 minutes. Washing with HBSS-2% FCS buffer was repeated twice, and the pellet was resolved in 1 ml Perm/Wash. 20,000 cells were acquired on the flow cytometer and saved in list mode.

Light microscopy (IV)

Kidney, spleen and liver tissues were examined by light microscopy using paraffin-embedded material cut and stained with paraaminosalicylic acid (PAS) and PAS-silver methenamine (PASM) as described by Eneström and Hultman [Enestrom and Hultman, 1984]. The type of glomerular damage was assessed and the degree of cellular proliferation was scored as follows: 0, no proliferation; 1, slight proliferation; 2, moderate proliferation; 3, severe proliferation. The fraction of glomeruli showing irreversible damage due to hyaline-sclerotic obsolescence of the capillary loops was assessed. The tubulointerstitial damage (tubular atrophy, interstitial fibrosis and chronic inflammation) was scored as follows: 0, no damage; 1, slight damage; 2, moderate damage; 3, severe damage. The assessment was made without knowledge of treatment or other results.

Statistical procedure

Statistical analyses were performed using GraphPad (Software Inc.) and Minitab (Minitab Inc.). The non-parametric Spearman's rank correlation test was used to assess dose-response relationships. The comparison between survival in controls and thimerosal-treated mice was performed using Log-rank test. To assess differences between 3 or more groups, parameters were analyzed by the non-parametric Kruskal-Wallis test followed by Dunn's post test. Numerical values between two different groups were analyzed using non-parametric Mann-Whitney *U* test. Fisher's exact test was used for comparison of discrete variables. And Logistic regression was used to study dose-response relationships for localization of tissue deposits, as well as severe proteinuria and probability for spontaneous death. $P < 0.05$ was considered to be statistically significant.

RESULTS

Thimerosal-induced murine immunosuppression (II)

Treatment of female A.SW mice with 10 mg thimerosal/L (corresponding to 590 µg/kg/bw/day) indicated suppression of many immune parameters early on after onset of treatment. The mean splenic weight decreased by 13% after 2.5 days compared with the controls ($p>0.05$), but showed a statistically significant increase after treatment for 6 days or more. The mean total number of splenic lymphocytes was reduced with 65% ($p<0.05$) and 21% ($p>0.05$) compared with controls after 2.5 and 6 days, respectively. After treatment for 8.5 and 14 days the total number of lymphocytes was in the range of the controls, but showed a 76% increase after 30 days treatment ($p<0.05$). The mean number of splenic CD3⁺ (pan T) cells decreased by 34-55% and the mean number of splenic CD4⁺ (T-helper) cells by 33-56% during the first 14 days of treatment, which was statistically significant except for after 8.5 days. The mean number of CD19⁺ (B cells) showed a profile similar to that of the total number of lymphocytes with 58% ($p<0.05$) and 34% ($p>0.05$) decrease after 2.5 and 6 days, respectively, compared with the controls, values close to the controls after 8 and 14 days, and a significant increase after 30 days. The mean number of splenic cIgE⁺ and cIgG2a⁺ cells decreased by 40% and 49%, respectively, after 6 days treatment with thimerosal ($p<0.05$), while the number of splenic cIgG1⁺ cells did not decrease. However, the number of all three cIg⁺ cells increased after 8.5, 14, and/or 30 days. The mean serum IgG1, IgG2a, and IgE concentration were not significantly reduced after 6 days, but increased after 8.5-14 days. The polyclonal B-cell activation markers anti-ssDNA and anti-DNP abs showed a slightly reduced mean value, 12-28% respectively, after 6 days of treatment, but none of these alterations were significant.

Murine systemic autoimmunity induced de novo by thimerosal

Immunostimulation (I, II)

The expression of lymph node cytokine mRNA (II) of IL-2 and IL-15 was significantly increased after 2.5 days treatment with 10 mg/L thimerosal compared

with controls, but declined close to control values after 6 days, when the expression of IFN- γ and IL-4 mRNA was increased 2- and 7-fold, respectively. The IFN- γ expression showed an apparent cyclic pattern with a decline towards control values after 8.5 days, a renewed increase after 14 days, and a decline towards control values after 30 days. The IL-4 mRNA expression declined rapidly to only 50% increase after 8.5 days compared with the controls, but showed a steady increase after 14 and 30 days ($p < 0.05$) compared with the controls.

The mean splenic weight (II) went from a non-significant reduction compared with the controls after 2.5 days treatment with 10 mg thimerosal/L, to a 21% increase ($p < 0.05$) after 6 days, and a 62-71% after 8.5-30 days treatment ($p < 0.01$ except after 30 days). The mean total number of splenic lymphocytes (II), went from a 65% significant reduction ($p < 0.05$) after 2.5 days and a non-significant 21% reduction after 6 days, to values close to the controls after 8.5 and 14 days, and a significant increase after 30 days. The mean number of splenic CD3⁺ and CD4⁺ cells (II) went from a significant decrease during the first 14 days of treatment to an increase by 53% and 63%, respectively, after 30 days treatment compared with the controls. The mean number of CD19⁺ cells changed from a significant decrease after 2.5 and 6 days treatment to values close to the controls after 8 and 14 days, and to a 67% increase ($p < 0.05$) after 30 days. The mean number of cIgE⁺ cells (II), which was significantly decreased after 6 days treatment with thimerosal, showed a 6-fold increase after 8.5 days treatment ($p < 0.05$) and a doubling after 14 and 30 days compared with the controls. Splenic cIgG2a⁺ cells (II) showed the same profile although the increase was limited to 150% ($p < 0.05$) and occurred only after 14 days treatment. The mean number of cIgG1⁺ cells (II), which was not reduced after 6 days treatment, showed a 10- and 5-fold increase, respectively after 8.5 and 14 days treatment ($p < 0.01$). The mean serum IgG1, IgG2a, and IgE concentrations went from no differences compared to the controls after 6 days thimerosal treatment to an increase during the interval of 8.5-30 days treatment in the following way (II). The serum IgE concentration increased 15- and 30-fold after 8.5 and 14 days treatment ($p < 0.05$), respectively. The IgG1 concentration increased 48% and 8% after 8.5 days and 30 days treatment, respectively ($p < 0.05$). The mean IgG2a concentration showed a 24% increase but only after 14

days treatment ($p < 0.01$). For longer time treatment with 10 mg thimerosal/L was associated with the following alterations of the serum Ig concentrations in the A.SW mice (I). Serum IgE showed a significant increase ($p < 0.05$) after 42 days and a non-significant increase after 70 days, and serum IgG1 showed a significant increase ($p < 0.05$) after both 42 and 70 days treatment. Serum IgG2a was increased neither after 42, nor after 70 days treatment.

Assessment of polyclonal B-cell activation using serum IgM and serum anti-DNP- and anti-ssDNA Abs showed after treatment with 10 mg thimerosal/L for 10 days a modest but significant increase in all three parameters (I), and after treatment for 14 days with the same dose in another study (II) a 60% increase for the two parameters assessed, anti-DNP- and anti-ssDNA abs, although the increase was statistically significant only for the latter ($p < 0.05$). Treatment for 42 days with 10 mg thimerosal/L caused a significant increase of all three parameters (I), and an increase was also seen after 70 days treatment for anti-ssDNA abs ($p < 0.01$) and serum IgM ($p < 0.05$) (I).

Autoantibodies (I, II, III)

Antinuclear antibodies

All mouse strains of the H-2^s haplotype (A.SW, SJL, and B10.S) exposed to sufficient doses of thimerosal developed serum IgG antinuclear autoantibodies which decorated the nucleoli with bright granules (“clumpy” pattern) [Pollard *et al.*, 1997], stained the condensed chromosomes in dividing cells weakly, and stained 2-6 dots in the nucleoplasm (I, II, IV). Immunoblotting showed that these ANoA positive sera generally reacted with a 34-kDa nucleolar protein identified as fibrillarin (II). The above strains, which share the H-2^s haplotype but have different non-H-2 genes, showed at a dose of 10 mg thimerosal/L a much higher titer in the A.SW strains compared with the B10.S and SJL strain (II), underlining the modifying effect also of non-H-2 genes for the induction of ANoA/AFA. By using A.TL and B10.TL mice, which share the background genes with the A.SW and B10.S strain respectively, but carry other genes than *s* in most H-2 loci (*k* and *d*), the main susceptibility to development of ANoA after treatment with thimerosal was localized to H-2 (II), which

is in accordance with observations using inorganic Hg [Hultman *et al.*, 1992; Kono *et al.*, 2001].

A dose of 2 mg thimerosal/L drinking water (118 µg Hg/kg bw/day), did not cause ANoA after 8 days treatment, but 60% of the mice showed ANoA after 14 days, exclusively of the IgG2a isotype (III). After 21 days treatment 50% of the mice showed ANoA which consisted of all IgG isotypes, although the IgG2a isotype dominated. A dose of 10 mg thimerosal/L (589 µg Hg/kg bw/day), did not cause ANoA after 8 days, but after 10 days when 7/10 mice showed IgG2a ANoA, and two of these mice also showed IgG1 ANoA. After 12 days 80% of the mice given 10 mg thimerosal/L showed ANoA of the IgG1 and IgG2a isotype. After 14 days 100% of the mice showed ANoA of the IgG2a and IgG1 isotype and lower titers of the IgG2b and IgG3 isotype were also present. In comparison, 8 mg HgCl₂/L (148 µg Hg/kg bw/day), which was found to give the fastest induction of ANoA using inorganic Hg, ANoA appeared in occasional mice after 4 days, in 30% after 8 days, and in all mice after 10 days treatment, while a dose of 3 mg HgCl₂/L (56 µg/kg bw/day) and 25 mg HgCl₂/L (463 µg/kg bw/day) caused a slower induction, ANoA first being observed after 14 and 30 days, respectively (III).

The titer of IgG ANoA in A.SW mice treated with thimerosal (2 and 10 mg/L) for 14 days was in order of IgG2a>IgG1>IgG2b>IgG3 (III).

Other autoantibodies (I)

Treatment with a high dose of thimerosal (40 mg/L) caused a statistically significant increase of antichromatin IgG antibodies after 10 days compared with controls, and all dose levels (1.25 – 40 mg/L) caused a statistically significant increase of ACA after 42 and/or 70 days treatment. However, while statistically significant the absolute increase in ACA was slight (< 0.1; OD at 405 nm) compared with controls (0.015-0.065), and the biological significance is uncertain.

Tissue immune-complex deposits

Following a dose of 2.5 mg/L, thimerosal caused granular deposits of IgG in the renal glomerular mesangium (which was not seen in the controls), and the increase was statistically significant ($P < 0.01$) when the number of positive mice were compared with the controls. Controls as well as thimerosal-treated mice showed granular deposits of IgM in the glomerular mesangium with a mean titer which was higher in groups given 5 mg/thimerosal/L or more for 70 days ($p > 0.05$). The mean titer of granular deposits of C3c in the mesangium was higher in all groups given 2.5-40 mg thimerosal/L compared with the controls ($p > 0.05$). Granular deposits of IgG and C3c were not seen in the splenic and renal vessel walls of the controls, but developed after at treatment with at least 2.5 and 5 mg thimerosal/L, respectively, for 70 days ($p < 0.05$) (I). After treatment for 30 days with 10 mg thimerosal/L granular mesangial as well as renal and splenic vessel wall deposits of IgG had developed (II). In contrast, the same dose for 14 days did not result in such deposits (III).

Dose-response relationships in murine - systemic autoimmunity induced de novo by thimerosal

Observation in A.SW mice given a range of doses of thimerosal (1.25-40 mg/L) in drinking water for 14 (II), 10, 42 or 70 (I) days showed a dose-response correlation for most of the autoimmune parameters (Table 1). The effect of several immune parameters in thimerosal-induced, systemic murine autoimmunity were only examined at a single dose (10 mg/L drinking water - 590 μg Hg/kg bw/day) (II). These parameters included significantly increased mRNA expression of IL-2 and IL-5 after 2.5 days treatment, IFN- γ and IL-4 after 6, 14, and for IL-4, also 30 days treatment. Phenotypic expressions of immunostimulation were also observed at this doses: the splenic weight was significantly increased after 6, 8.5, and 14 days treatment, total number of splenic lymphocytes, CD3⁺, CD4⁺, and CD19⁺ cells after 30 days treatment. The number of cIgG1⁺ cells was significantly increased after 8.5 and 14 days, cIgE⁺ cells after 8.5 days, and cIgG2a⁺ cells after 14 days (II). For many of the parameters treatment time and not only the daily dose also affected the response as indicated in

table 1. While treatment for 10 days was associated with an increase of IgE and IgG2a, most parameters required 42 or 70 days. The markers for polyclonal B-cell activation were significantly increased after 10, 42, and 70 days treatment, but the increase was slight in absolute values making the biological significance uncertain (I).

Table 1. Dose-response relationships for different parameters in thimerosal-induced de novo systemic autoimmunity in A.SW mice.

| Parameter | NOAEL ($\mu\text{g Hg/kg bw/day}$) | LOAEL ($\mu\text{g Hg/kg bw/day}$) | Time ^a (days) | Dose-response | | Way of admin. | Paper no. | |
|----------------------------------|---|---|-----------------------------|---------------|-----------------|------------------|-----------|----|
| | | | | Y/N | Range (mg/L) | | | |
| AFA | 74 | 147 | 42 | Y | 0-40 | 42, 70 | O | I |
| ACA | <74 ^b | 74 ^c | 70 | Y | 0-40 | 42, 70 | O | I |
| Serum IgM | 74 | 147 | 10 | Y | 0-40 | 10, 42, 70 | O | I |
| Anti-ssDNA abs | 74 | 590 ^d | 10, 70 | Y | 0-40 | 10, 42, 70 | O | I |
| Anti-DNP abs | 295 | 590 | 10 | Y | 0-40 | 10, 42, 70 | O | I |
| Serum IgE | 147 | 295 | 10 | Y | 0-40 | 10 | O | I |
| Serum IgG1 | 147 | 295 | 70 | Y | 0-40 | 42, 70 | O | I |
| Serum IgG2a | 295 | 590 | 14 | Y | 0-40 | 42, 70 | O | II |
| Renal mesangial IgG deposits | 74 | 147 | 70 ^e | Y | 0-40 | 70 | O | I |
| Kidney vessel wall IgG deposits | 74 | 295 | 70 ^e | Y | 0-40 | 70 | O | I |
| Splenic vessel wall IgG deposits | 74 | 147 | 70 ^e | Y | 0-40 | 70 | O | I |

NOAEL, No observed adverse effect level

LOAEL, Lowest observed adverse effect level

Y/N, Yes/No

AFA, antifibrillar antibodies

O, Perorally in the drinking water

ACA, antichromatin antibodies

^a Figures denote observations for NOAEL and LOAEL

^b The lowest dose examined

^c Weak increase in absolute titers, biological significance uncertain

^d Mice given 147, but not 295 $\mu\text{g Hg/kg bw/day}$ showed a statistical significant increase ($p < 0.05$) after 70 days.

^e Only examined at sacrifice after 70 days treatment

Organic mercury compounds - primary autoimmunogen substances?

The studies of thimerosal with regard to de novo induction of murine, systemic autoimmunity pointed to many similarities with inorganic Hg with regard to genetic and immunological requirements as well as the outcome assessed by immunological parameters for immunostimulation and autoimmunity (I, II). Since inorganic Hg is rapidly formed from the metabolic conversion in the body of the EtHg in thimerosal, the autoimmunogen effect of thimerosal treatment might have been related to a larger or smaller degree to the metabolically formed inorganic Hg. On the other hand, another organic Hg compound, MeHg induces, like primary treatment with inorganic Hg and thimerosal (EtHg), ANoA/AFA in genetically susceptible mice. However, MeHg-induced autoimmunity lacks several features of HgIA such as the strong immunostimulation and the development of IC deposits even using equimolar doses of Hg as compare with thimerosal and HgCl₂ [Haggqvist *et al.*, 2005]. This indicated primary and/or modifying effect of organic Hg compounds on the autoimmune response. To study primary autoimmunogenic effect of thimerosal (EtHg) and MeHg, we took two approaches. First, we examined if sufficient amount of inorganic Hg is formed early on during thimerosal and MeHg treatment in order to cause the autoimmune syndrome, by assessing the accumulation of inorganic Hg in the kidneys of thimerosal- and MeHg-treated mice and comparing these concentrations with the renal threshold dose of inorganic Hg for induction of the autoimmune syndrome using HgCl₂. The renal concentration of inorganic Hg was chosen since it is the best indicator of the body burden of inorganic Hg [WHO, 1991]. Secondly, we tried to establish if the organic Hg compounds might have qualitatively different autoimmunogen effects compared with inorganic Hg. For studying this effect we focused on the IgG isotype of the ANoA/AFA which rapidly developed during treatment with inorganic and organic Hg substances, and which should therefore accurately reflect the effect of the mercury compounds on the immune system, for example on the type of T-helper cells activated, the IgG2a isotype reflecting activity of T-helper cells of type 1 (Th1), and the IgG1 isotype T-helper cells of type 2 (Th2).

Treatment with low dose of HgCl₂ in A.SW mice by 0.8 mg/L drinking water (15 µg Hg/kg bw/day) for up to 21 days was not associated with any ANoA response, and the kidney concentration of Hg²⁺ was 0.32 and 0.36 µg/g after 8 and 14 days, respectively. Sixty percent of mice given 1.5 mg HgCl₂/L (28 µg Hg/kg bw/day) for 14 days developed ANoA, and kidney Hg²⁺ was 0.62 µg/g after 5 days and 0.53 µg/g after 8 days. Even with an optimal dose of 8 mg HgCl₂/L ANoA was not observed in any mouse until after 4 days treatment and 8 days treatment was necessary to induce ANoA in a majority of the mice. Since the dose threshold for primary induction of ANoA is ca. 1 mg HgCl₂/L (22 µg Hg/kg bw/day) [Hultman and Nielsen, 2001] and 50% of the steady state for Hg²⁺ is reached after 2 days [Hultman and Nielsen, 1998], the threshold of Hg²⁺ for induction of ANoA is likely to be reached within 24 hours using an 8-fold higher dose of HgCl₂ (8 mg/L). Therefore, the Hg²⁺ concentration in the 1.5 mg/L group after 7-8 days treatment must have been above the induction threshold in order to result in ANoA after 14 days. This means that the threshold for induction of ANoA correspond to a renal Hg²⁺ concentration of more than 0.32 µg/g, but less than 0.53 µg/g. A dose of 0.8 mg MeHg/kg bw every third as sc injections day and 2 mg thimerosal/L drinking water, which resulted in ANoA after 14-21 days, corresponded to a renal tissue Hg²⁺ concentration of 2.2 and 3.4 µg/g, respectively, after 8 days which is clearly above the threshold for eliciting ANoA with inorganic Hg. Therefore, the ANoA induced after treatment with the lowest doses of MeHg and thimerosal used in study III could have resulted entirely from the effect of the metabolically formed Hg²⁺.

What about qualitative differences between inorganic and organic Hg compounds? The lower doses of HgCl₂ (1.5 and 3 mg/L) caused a dominance of the Th1-dependent IgG2a isotype after 14 days treatment, which persisted after 30 days although with increased variation between the animals. A dose of 8 mg HgCl₂/L caused an initial dominance of the IgG2a isotype which evolved into a subsequent increase of the Th2-dependent IgG1 isotype with time, leading to a balanced index between Th1/Th2 (IgG2a/IgG1 index nil). A higher dose of HgCl₂ (25 mg/L) caused already from onset of ANoA development after 10 days a balanced IgG2a/IgG1 index, which developed into a modestly negative index (IgG1-Th2-dominance) after 12-14 days treatment.

The IgG2a/IgG1 index was different in MeHg- and EtHg- treated mice, compared with HgCl₂-treated mice, since the Th1 pattern dominated already during the earliest induction of ANoA, and continued with a Th1-skewing also after 30 days treatment with the organic compounds.

These observations of a shift in the index in favor of a Th1 phenotype during treatment with MeHg and EtHg compared with HgCl₂ indicate a qualitative difference between the way organic and inorganic Hg compounds interact with the immune system during induction of ANoA. Whether this is caused by differences in activation or suppression of Th1- and Th2-cells is not revealed from these studies.

Thimerosal induced acceleration of spontaneous autoimmune murine disease

Apart from the ability of inorganic Hg to induce de novo systemic autoimmunity in mouse strains, with different expression dependent on genetic factors, it has recently been shown that inorganic Hg may accelerate the systemic autoimmune disease which occurs in certain strains such as ZBWF1, BXSB, MRL^{+/+}, MRL^{lpr/lpr} [al-Balaghi *et al.*, 1996; Abedi-Valugerdi *et al.*, 1997; Pollard *et al.*, 1999; Pollard *et al.*, 2001; Pollard *et al.*, 2005] in a strain-specific manner and not related to H-2 [Pollard *et al.*, 2001]. However, this ability has not been studied for organic Hg compounds. Groups of ZBWF1 mice were therefore exposed to different doses of thimerosal (0.156-5 mg/L) for up to 25 weeks and the development of a number of disease parameters were compared for the thimerosal-treated mice and the untreated controls by regular blood samplings and by tissue sampling after spontaneous death or at sacrifice.

Features during the first 7 weeks

ANA with a homogeneous or finely speckled pattern began to appear in the two highest dose groups (2.5 and 5 mg/L) after 4 weeks. ANA were significantly more frequent (p<0.01) in these groups after 7 weeks compared with the controls, when the presence of ANA also showed a significant correlation with the dose of thimerosal (p<0.05; Spearman's rank test). Some of the mice receiving the highest dose of thimerosal (5 mg/L – 295 µg Hg/kg bw/day) and corresponding control mice were

sacrificed after 7 weeks. While controls showed only granular mesangial IgM deposits, the thimerosal-treated mice showed granular mesangial deposits of IgM, IgG1, IgG2a, IgG3 (all $p < 0.05$) and to some extent also IgG2b; the IgG1 deposits dominated in terms of the titer. The controls lacked any immune deposits in the splenic and renal vessel walls, while the thimerosal-treated mice showed granular deposits of IgG1 and C3c in the renal vessel walls and of IgG1, IgG2b, IgG3 and C3c in the splenic vessel walls. The serum IgE concentration was significantly increased after 10 days treatment with 5 mg thimerosal/L compared with the controls ($p < 0.001$), but the increase was a modest 3-fold. However, there was a significant relationship between the IgE concentration and the dose of thimerosal after 10 days ($p = 0.0067$; Spearman's rank test).

Features after 7-25 weeks

The subsequent development of the autoimmune features until sacrifice after 25 weeks or spontaneous death was characterized by a gradual increase of the frequency of mice with ANA of a homogeneous or finely speckled pattern, as well and the titer of ANA, also in mice given lower doses of thimerosal and in controls, but both the frequency and the titer of ANA showed a significant correlation with the dose of thimerosal after 13 weeks ($p < 0.05$; Spearman's rank test). A majority of the mice showed ANA after 16 weeks, and the cumulated incidence of ANA with a homogeneous, finely speckled and coarsely speckled pattern was 89-100% at the end of the study. However, from 16 weeks and further on there was no significant correlation between treatment with thimerosal and ANA development. Part of the ANA in controls as well as thimerosal-treated mice were due to IgG antibodies to double-stranded DNA (anti-dsDNA abs), and they may have contributed to the accelerated development of ANA especially after 13 weeks in the 1.25 and 2.5 mg/L group, but from 16 weeks and on there was no significant correlation between the dose of thimerosal and the presence of anti-dsDNA abs; the cumulated incidence of anti-dsDNA abs at the end of the study was 70-100% in the different groups. Antichromatin antibodies (ACA) of the IgG class also contributed to the ANA observed in thimerosal-treated mice as well as the controls.

The titer started to increase substantially after 13 weeks in all groups including the controls, and then rose gradually with large individual variations until after 22 weeks, when the titer plateaued or decreased for the last 3 weeks. Although the mean ACA titer in the three highest dose groups after 13 weeks was higher than in the lower dose groups and in the controls, there was in general no significant difference in ACA titer between thimerosal-treated mice and controls, and no correlation between the ACA titer and the dose of thimerosal.

Features of immune stimulation in the form of polyclonal B-cell activation assessed as the titer of anti-ssDNA and anti-DNP antibodies started to increase after 7 weeks and continued to increase until end of the study, but the titers were not significantly different in the thimerosal-treated mice compared with the controls. The serum IgG1 and IgG2a concentration gradually increased in both controls and thimerosal-treated mice but there was no consistent correlation with thimerosal treatment.

The main features of thimerosal-induced alteration of the spontaneous systemic autoimmune disease in ZBWF1 mice after 22-25 weeks treatment concerned the localization of the immune deposits in glomerular structures, and the morphological, functional, and mortality consequences of this effect, as well as the development of renal and splenic vessel wall immune deposits at a dose of Hg lower than hitherto observed.

After 22-25 weeks heavy deposits of IgG, all IgG subclasses, IgM and C3c were present in glomerular structures in controls as well as thimerosal-treated mice irrespectively of the dose. The mean titer of glomerular IgG1 deposits in the 5 mg/L group was higher compared with the controls. However, the most important effect was a shift in the localization of the glomerular deposits. While the deposits were predominantly localized along the capillary walls in the controls and in mice given the two lowest doses of thimerosal (0.156 and 0.313 mg/L), the mice in the two highest dose groups (2.5 and 5 mg/L) showed a predominantly mesangial pattern. The difference in glomerular localization between the two highest dose groups on the one hand and the two lowest dose groups and the controls on the other hand was significant ($p < 0.05$). In addition, the correlation between the dose of thimerosal and the localization of IgG and C3c deposits in the glomeruli was significant ($p < 0.05$). The

morphological alteration in glomeruli assessed as the degree of endocapillary cell proliferation showed a significant inverse relationship with the dose, so that higher doses were associated with less proliferation ($p < 0.05$; Spearman's rank test). In addition, the fraction of irreversibly damaged glomeruli as well as the degree of chronic tubulointerstitial damage showed a significant correlation ($p < 0.001$) with the degree of endocapillary cell proliferation.

With regard to functional effects of thimerosal-treatment, the cumulated incidence of mice with high-grade proteinuria (>1.5 g/L) showed a significant inverse correlation with the dose of thimerosal ($p < 0.05$). Furthermore, high-grade proteinuria was also significantly more frequent in mice with capillary wall deposits, more severe endocapillary cell proliferation, a high fraction of irreversibly damaged glomeruli, and a high grade of chronic tubulointerstitial damage ($p < 0.01$). Finally, there was a correlation between high-grade proteinuria and spontaneous death between 19 and 25 weeks ($p < 0.05$).

Another important feature of thimerosal-induced alteration of the spontaneous systemic autoimmune disease in ZBWF1 mice was induction of renal and splenic vessel wall deposits. While occasional control mice showed immune deposits (IgG2a and C3c) in the renal vessel walls, they showed no deposits of any IgG isotype or C3c in the splenic vessel walls. Mice treated with 0.313-5 mg thimerosal/L showed granular renal vessel wall deposits of IgG1, IgG2a, and IgG2b with a dominance of IgG1 ($p < 0.05$) and accompanying C3c deposits. The titer of IgG1 and IgG2a showed a significant relationship with the dose of thimerosal ($p < 0.05$). Granular splenic vessel wall deposits of IgG1, IgG2a, and C3c were found in mice given all different doses of thimerosal, also including the lowest dose (0.313 mg/L), and vessel wall deposits were seen in all mice given a dose of 0.625 mg/L or more. Furthermore, the splenic IgG1, IgG2a, and C3c vessel wall deposits showed a significant relationship with the dose of thimerosal ($p < 0.05$). With regard to morphological alterations no signs of vasculitis were observed in the vessel walls.

The dose-response relationships for the effect of thimerosal on spontaneous autoimmunity in ZBWF1 mice are summarized in Table 2.

Table 2. Dose-response relationships for acceleration of autoimmune parameters in ZBWF1 mice after treatment with thimerosal (paper IV).

| Parameter | NOAEL ($\mu\text{g Hg/kg bw/day}$) | LOAEL | Time ^a (weeks) | Dose-response | | Way of admin. |
|---|---|-------|------------------------------|---------------|-----------------|------------------|
| | | | | Y/N | Range (mg/L) | |
| ANA | 74 | 147 | 7 | Y | 0-5 | 7, 13 O |
| Serum IgE | 147 | 295 | 1.5 | Y | 0-5 | 1.5 O |
| Serum IgG1 | 9 | 18 | 19 | N | - | - O |
| Serum IgG2a | - | - | - | Y | 0-5 | 16 O |
| Redistribution of glomerular immune deposits | 74 | 147 | 25 | Y | 0-5 | 25 O |
| Kidney vessel wall deposits | 9 | 18 | 25 | Y | 0-5 | 25 O |
| Splenic vessel wall deposits | <9 ^b | 9 | 25 | Y | 0-5 | 25 O |

NOAEL, No observed adverse effect level

LOAEL, Lowest observed adverse effect level

Y/N, Yes/No

ANA, antinuclear antibodies

O, Perorally in the drinking water

IC, Immune-complex

^a Figures denote observation for NOAEL and LOAEL

^b The lowest dose studied

DISCUSSION

The active component of thimerosal

As described in the introduction thimerosal contains 49.6% Hg by weight as ethyl mercury (EtHg), which is rapidly released from the non-mercurial component, thiosalicylic acid, in the tissues. The exact events in the breakdown of EtHg is unknown, but the fraction of Hg^{2+} in the tissues increased rapidly after thimerosal treatment: 20-25% in the kidneys after 8-14 days of thimerosal (118 μg Hg/kg bw) (III), after 590 μg Hg/kg bw a peak of 41% in the kidneys after 14 days and 29% after 30 days, when the mesenterial lymph nodes showed 30% (II). These figures tallies with the fraction of Hg^{2+} being 27% (kidney) and 13% (lymph nodes) in thimerosal-treated mice given 590 μg Hg/kg bw/day for 6 days [Qvarnstrom *et al.*, 2003]. Monkeys receiving a total of 80 μg Hg/kg bw as thimerosal during 21 days showed a peak fraction of 86% Hg^{2+} in the brain [Burbacher *et al.*, 2005], while treatment of monkeys with 2 and 10 μg Hg/kg bw/day for 6 months resulted in a renal Hg^{2+} fraction of 90% and 77%, respectively [Blair *et al.*, 1975], and in a human case 3 months exposure resulted in a Hg^{2+} fraction of 51-69% [Clarkson, 1972]. A significant dose-response relationship was recently established for thimerosal and apoptotic cell death in Jurkat T-cells [Makani *et al.*, 2002], as well as toxic tissue reactions with severe inflammation after injections of thimerosal [Uchida *et al.*, 1994]. Since thiosalicylic acid, the non-mercury component of thimerosal, caused neither apoptotic cell death [Makani *et al.*, 2002] nor toxic tissue reactions [Uchida *et al.*, 1994], the effects of thimerosal on cell viability and tissue inflammation is due to the mercurial component, EtHg.

Induction of de novo systemic autoimmunity in mice by thimerosal

Thimerosal-induced antinuclear antibodies

Treatment with thimerosal, containing EtHg, was able to induce antinucleolar antibodies with fibrillarin as the main target in genetically susceptible mouse strains (I,

II, III), a characteristic shared with to MeHg [Hultman and Hansson-Georgiadis, 1999; Haggqvist *et al.*, 2005], and inorganic Hg in the form of HgCl₂ [Hultman *et al.*, 1996] and metallic mercury (vapor) [Warfvinge *et al.*, 1995]. Linkage of the susceptibility to certain H-2 haplotypes, specifically the H-2IA locus (II), and a modulating effect of non-H-2 genes, was also shared between thimerosal (II), MeHg [Hultman and Hansson-Georgiadis, 1999], and HgCl₂ [Hultman and Johansson, 1991]. The mechanism underlying induction of ANoA/AFA is not firmly established, but the best and partly proven hypothesis is modified processing of the fibrillar in due to alterations in proteases caused by Hg-induced cell death [Pollard *et al.*, 2000]. This leads to formation of neopeptides of fibrillar, which are recognized as foreign by T-cells [Kubicka-Muranyi *et al.*, 1995]. This might be supplemented by subsequent epitope spreading making epitopes also in unmodified fibrillar (peptides) a target for T-cells [Kubicka-Muranyi *et al.*, 1996]. Elucidating the molecular-biochemical effects of thimerosal on fibrillar was not the aim of this thesis, but it should be noted that thimerosal has a strong cytotoxic effect [Makani *et al.*, 2002; Baskin *et al.*, 2003] which indicates that it might have the same modifying effect of proteases as inorganic Hg [Pollard *et al.*, 1997; Pollard *et al.*, 2005]. However, since mercuric mercury (Hg²⁺) is rapidly formed following thimerosal treatment [Magos, 2003; Qvarnstrom *et al.*, 2003] also evidenced in these studies (II, III), a distinct possibility is that thimerosal (EtHg) exerts part or all of its autoimmunogen effect by means of the formed Hg²⁺. The slightly delayed ANoA/AFA induction from treatment with thimerosal as compared with HgCl₂ (III) is an indication that the autoimmunogen effect of thimerosal may actually be due to metabolically formed Hg²⁺ (III). However, another possibility is that the early immunosuppressive effect of thimerosal (II), as opposed to HgCl₂ [Johansson *et al.*, 1997; Johansson *et al.*, 1998], delayed the autoimmunogen effect.

The dose-response relationship observed for inorganic Hg with regard to ANoA/AFA induction [Hultman and Nielsen, 2001] also applied for thimerosal (I). Furthermore, the absolute requirement for T-cells [Hultman *et al.*, 1995], the T-cell costimulatory factor CD28 [Bagenstose *et al.*, 2002; Pollard *et al.*, 2005], and the IFN- γ /IFN- γ -

receptor pathway but not IL-4 [Bagenstose *et al.*, 1998; Kono *et al.*, 1998] for induction of ANoA/AFA by inorganic Hg was also shared by thimerosal (II).

Cytokine induction by thimerosal

Thimerosal treatment caused, like inorganic Hg [Haggqvist *et al.*, 2005], a rapidly increased mRNA expression in lymph nodes of cytokines such as IL-2, IFN- γ , IL-15, IL-4 after onset of treatment (II), although the increase of IFN- γ mRNA was more forceful and protracted than after treatment with inorganic Hg. However, the lack of IL-6 had a differential effect on ANoA induction using thimerosal and Hg²⁺, since the ANoA response was abolished after treatment with thimerosal (II) but preserved after Hg²⁺ treatment [Pollard *et al.*, 2005]. IL-6-deficient B10.S (H-2^s) mice treated with Hg²⁺ showed a loss of the ACA response [Pollard *et al.*, 2005], similar to observations in murine pristane-induced autoimmunity [Richards *et al.*, 1998], which might be related to the fact that one of the inducers for IL-6 is the IFN- γ / IFN- γ R pathway [Boehm *et al.*, 1997]. However, the explanation for the different dependency of ANoA induction in IL-6 deficient mice between thimerosal and Hg²⁺ remains unknown.

The mechanism for induction of cytokines by Hg are poorly understood, but recent studies have shown that absence of the genes for molecules which regulate, and perhaps mainly augment [Szabo *et al.*, 2003], IFN- γ expression (IL-12p35, IL-12p40, STAT4, ICE), do not significantly influence induction of HgIA by Hg²⁺ [Pollard *et al.*, 2005]. Since the genes of IFN- γ , IFN- γ R, and interferon response factor 1 (IRF-1) (the downstream primary response gene for IFN- γ [Boehm *et al.*, 1997] are needed for induction of HgIA, Hg may directly induce IFN- γ , IFN- γ seems however to be needed in low concentrations, as illustrated by the mainly intact HgIA response in mice with approximately 50% protein production (heterozygous for the IFN- γ gene) [Kono *et al.*, 1998]. The above studies were done in lymphoid tissues after Hg treatment; to what extent cytokines were locally produced in the lymphoid tissue of the gastrointestinal tract and cutaneous tissues after oral and subcutaneous treatment, respectively, is uncertain. However, there are preliminary data of upregulation of

cytokines such as TNF- α and IL-1 β , IL-6 and IL-10 in cutaneous tissues shortly after HgCl₂ injection although the contribution to HgIA is unknown [Pollard *et al.*, 2005]. For IL-4, there are indications of a direct effect of Hg on immune cells. A rat cell line [Wu *et al.*, 2001], murine mast cells [Walczak-Drzewiecka *et al.*, 1999], and a murine T-cell hybridoma [Badou *et al.*, 1997] showed increased IL-4 mRNA or protein expression after exposure to HgCl₂. In the latter study it was shown that HgCl₂ could directly activate protein kinase and Ca²⁺ influx through L-type calcium channels.

Thimerosal-induced lymphoproliferation – relation to cytokines

Thimerosal shared with inorganic Hg [Johansson *et al.*, 1997; Johansson *et al.*, 1998] the ability to cause lymphoproliferation, assessed as an increase in splenic weight, number of splenic lymphocytes, number of splenic T-and B-cells, cIg-secreting cells (II), and serum Ig concentrations (I, II, III). However, the signs of immunostimulation were delayed compared with the effect of inorganic Hg [Johansson *et al.*, 1997; Johansson *et al.*, 1998], which might be due either to a need for thimerosal to be converted to Hg²⁺ in order to exert immunostimulation, or to the opposing effect of the immunosuppression caused by thimerosal during the first 1-2 weeks after onset of treatment (II). Interestingly, the increased expression of the cytokines IL-2, IFN- γ , IL-15, and IL-4 was not significantly delayed compared with HgCl₂ despite the initial reduction of T-and B-cells [Haggqvist and Hultman, 2001]. This might be explained by activation of a fraction of the T-cells in the lymph nodes, which would result in increased mean mRNA expression despite a reduction in the total number of lymphocytes/T-cells. Another explanation which might be considered is a difference in cytokine expression and lymphoproliferation between the mesenteric lymph nodes (in which the cytokine mRNA expression was assessed), and the spleen (in which the lymphoproliferation was assessed). However, a correlation was previously found between the cytokine expression in lymph nodes and spleen, but the spleen shows a lower absolute increase in expression than the lymph nodes [Haggqvist and Hultman, 2001]. Due to the small volume of mesenteric lymph nodes they can however not be

used for assessment of both cytokine mRNA expression and analysis of lymphoproliferation by flow cytometry.

Thimerosal-induced systemic immune-complex deposits

Thimerosal was able to induce glomerular mesangial and systemic vessel wall deposits of Ig and complement factors in a granular pattern, strongly indicating the presence of immune-complex deposits (I, II, III). The deposits were not qualitatively different from those induced by inorganic Hg [Hultman *et al.*, 1996].

The autoimmunogen effect of organic Hg species

Some qualitative differences between de novo systemic autoimmunity induced by thimerosal- and HgCl₂ have already been mentioned, such as a stronger and more protracted increase of IFN- γ (II), differential need for IL-6 with regard to ANoA/AFA induction (II), and delayed immunostimulation and ANoA/AFA induction (III). Features seen after primary exposure to HgCl₂ such as a strong increase of IL-4 mRNA, induction of IL-2 and IFN- γ , polyclonal B-cell activation, and systemic IC deposits were not seen after treatment with MeHg [Haggqvist *et al.*, 2005].

In paper III we tried to more closely analyze the autoimmunogen effect of thimerosal and MeHg compared with HgCl₂, to discern a possible primary autoimmunogen effect of the organic Hg species thimerosal (EtHg) and MeHg. The first approach was to assess if sufficient Hg²⁺ was formed in the body during treatment with thimerosal or MeHg to account for induction of ANoA/AFA. The lowest doses of thimerosal and MeHg employed in this study both caused a body burden of Hg²⁺, assessed as the renal Hg²⁺ concentration, above the threshold for primary induction of ANoA/AFA by HgCl₂ within the time frame of the induction. Therefore, with the doses of thimerosal and MeHg used in these studies, Hg²⁺ formed during decomposition of thimerosal could account for the observed autoimmunogen effect from a dose-response point of view. However, this does not exclude that the organic species may have contributed to the autoimmunogen effect. Further studies with lower doses of thimerosal and MeHg, closely tuning in the threshold for induction of ANoA/AFA, with simultaneous

speciation of Hg in renal and lymphoid tissues could possibly reveal if there exists a dose of these organic Hg species which leads to ANoA/AFA without reaching the threshold dose of tissue Hg^{2+} causing ANoA/AFA.

In addition to these quantitative aspects, we examined if there were any qualitative differences in the autoantibody response between thimerosal, MeHg and HgCl_2 . We focused on the IgG isotype of ANoA, which should accurately reflect the effect of the different Hg species on the cells of the immune system during the induction period. We were particularly interested in T-helper cells, since induction of ANoA/AFA seems to be a cognate, antigen-dependent, MHC class II-dependent (auto)immune (antigen-dependent) response. In analogy with studies on the T-helper cell response after immunization [Hovden *et al.*, 2005], the IgG isotype of the ANoA was used as an indication of the activation of Th1 and Th2 cells due to the association of IgG1 with Th2 cells [Kuhn *et al.*, 1991], and IgG2a with Th1 cells [Huang *et al.*, 1993]. We found that after HgCl_2 -treatment there was a dose- and time-dependent pattern with lower doses favoring a persistent IgG2a/Th1-dominance, while higher doses with time, or in the case of sufficiently high doses, already from onset, caused a more balanced or even IgG1/Th2-dominated response. In contrast, organic Hg species favored an IgG2a/Th1 response with little influence of dose or time. The findings indicate a qualitative difference between the autoimmunogen effect of organic and inorganic Hg species.

These observations corroborate with previous studies showing a dominance of Th1/IgG2a during treatment with organic Hg [Haggqvist *et al.*, 2005; Havarinasab *et al.*, 2005] compared with HgCl_2 [Haggqvist and Hultman, 2001]. However, the cause of this shifted balance might be different between MeHg and EtHg. While MeHg treatment (540 $\mu\text{g Hg/kg bw/day}$) caused a 2-3-fold increase of IL-4 mRNA and serum IgE [Haggqvist *et al.*, 2005], EtHg (590 $\mu\text{g Hg/kg bw/day}$) caused a 7-fold increase of IL-4 mRNA and 30-fold increase of IgE [Havarinasab *et al.*, 2005], which should be compared with a 15-fold increase of IL-4 mRNA and a 40-160-fold increase of IgE in after HgCl_2 treatment (360 $\mu\text{g Hg/kg bw/day}$) [Haggqvist and Hultman, 2001].

Furthermore, MeHg-treated mice did not show the Hg-induced increase in IL-2 and IFN- γ mRNA [Haggqvist *et al.*, 2005] reported after treatment with HgCl₂ [Haggqvist and Hultman, 2001], while these cytokines were slightly higher in the EtHg treated mice than after HgCl₂ treatment [Havarinasab *et al.*, 2005]. These observations may be interpreted as follows. The Th1-like dominance after MeHg treatment might have been caused by a weak activation and/or suppression of Th2 cells, while the Th1-like dominance in EtHg-treated mice might be due to a strong and/or more protracted increase of the Th1-associated cytokines IL-2 and IFN- γ [Havarinasab *et al.*, 2005]. The latter might in addition lead to reciprocal inhibition of Th2 cells.

The effect of inorganic Hg in determining activation of Th1 or Th2 cells has previously been studied. For example, HgCl₂ was shown to cause a Th2 skewing in vitro [Heo *et al.*, 1997]. Another observation indicates that intracellular levels of glutathione might be involved, since in rats GSH is required for Con A-mediated IFN- γ induction, and this correlates with the induction of IFN- γ by Hg in vivo [van der Meide *et al.*, 1993]. Furthermore, depletion of GSH in mice causes a decreased IFN- γ and increased IL-4 production upon in vitro stimulation of T-cells [Peterson *et al.*, 1998], and treatment of human T-cells in vitro with a GSH precursor caused an inhibition of IL-4 [Jeannin *et al.*, 1995]. These observations indicate that low levels of GSH favor a Th2 response and high levels of GSH support a Th1 response. Organic as well as inorganic Hg species cause a reduction of intracellular GSH, which has been associated with the cytotoxicity [Naganuma *et al.*, 1990; Sanfeliu *et al.*, 2003; James *et al.*, 2005], but it is unknown if organic species affect GSH levels differently than inorganic Hg.

Another possibility for explaining the difference between organic and inorganic Hg species on induction of Th1 and Th2 cells might be the differences in fine specificity of the autoantigen (fibrillar peptides), or differences in the release of IL-4 demonstrated to occur due to HgCl₂ from T-cells [Badou *et al.*, 1997], or mast cells in rats [Oliveira *et al.*, 1995], and mice [Walczak-Drzewiecka *et al.*, 1999], which may induce Th2-deviation.

Acceleration of spontaneous systemic autoimmunity by thimerosal (IV)

In paper IV the ability of thimerosal to accelerate and/or aggravate spontaneous systemic autoimmune disease was examined using a prototype model for spontaneous systemic autoimmunity, the (NZB X NZW)F1 hybrid [Theofilopoulos and Dixon, 1985]. During the first 13 weeks there was an acceleration in development of ANA, which tallies with previous studies in ZBWF1 mice [Pollard *et al.*, 1999], although antichromatin antibodies did not increase. However, anti-dsDNA abs contributed to the accelerated ANA development. After 7 weeks thimerosal had induced glomerular mesangial IgG deposits of various isotypes and of complement, which was not seen in the controls. These findings are similar to those of Abedi-Valugerdi *et al* (1997) using a 30 % higher dose of Hg as HgCl₂ [Abedi-Valugerdi *et al.*, 1997]. However, the previous study did not report renal or splenic granular vessel wall IC deposits. These findings in (IV) are indications of an accelerated spontaneous autoimmune reaction with regard to ANA, due to the very weak development of vessel wall deposits in the spontaneously developing ZBWF1 disease

After longer treatment than 13 weeks, the accelerating effect of thimerosal on ANA development was no longer discernible, and the hyperimmunoglobulinemia of IgG1 and IgG2a reported after treatment with a similar dose of Hg as in the present study but in the form of HgCl₂ [Pollard *et al.*, 1999] was not seen (IV), making thimerosal a much weaker inductor of such serological alterations. However, thimerosal had distinct effects on the systemic IC deposits which were only to some extent seen after HgCl₂ treatment. Thimerosal shared with HgCl₂ the tendency towards an increase of IgG1 immune deposits [Abedi-Valugerdi *et al.*, 1997], which indicates a skewing of the effect of thimerosal towards IgG1 and Th2. However, in other aspects the effect of organic and inorganic Hg differed. First, thimerosal in higher doses caused a relocalization of glomerular IC deposits, from the glomerular capillary walls to the mesangium which attenuated the histological damage, proteinuria and increased survival, in accordance with previous observations [Weening *et al.*, 2004] and attributed to better access to circulating inflammatory cells for capillary wall deposits [Nangaku and Couser, 2005]. Secondly, thimerosal induced in a dose-dependent

fashion heavy IC deposits in renal as well as splenic vessel walls which were present also using a very low dose. In renal vessel walls there was a dominance of IgG1, indicating a Th2-skewing effect although Th1-dependent isotypes such as IgG2a also were prevalent, which tallies with the primary pathogenetic role of IFN- γ in the pathogenesis of kidney lesions in ZBWF1 mice, and a contributing effect of Th2-associated cytokines such as IL-4 [Nakajima *et al.*, 1997]. While there were no indications of vasculitis, the functional effect of the vessel wall deposits is unknown.

Dose-response considerations for de novo induction of systemic autoimmunity by thimerosal and for the acceleration/aggravation of spontaneous autoimmunity

De novo induction of autoimmunity

The LOAEL and NOAEL for induction of the different autoimmune parameters in A.SW mice by thimerosal are presented in Table 1. The LOAEL was in the order AFA = renal mesangial and splenic vessel wall deposits = serum IgM < serum IgG1 and IgE = kidney vessel wall deposits < PBA = serum IgG2a. This order of the LOAEL is somewhat different from what has been found after treatment of this strain with HgCl₂, where AFA < serum IgE < tissue IC deposits [Hultman and Nielsen, 2001]. The latter order is similar to the order for HgIA induction in another H-2^s strain (SJL) after exposure to mercury vapor (metallic mercury – Hg⁰) [Warfvinge *et al.*, 1995]. The LOAEL for AFA by thimerosal in the drinking water to A.SW mice was 118 μ g Hg/kg bw/day (III), which should be compared with 11 μ g Hg/kg bw/day using HgCl₂ [Hultman and Nielsen, 2001], which is an 11-fold lower threshold dose after HgCl₂ exposure, making thimerosal a considerably weaker inductor of AFA de novo. The same observation was made for other HgIA parameters such as serum IgE, which had a LOAEL in A.SW mice of 295 and 37 μ g Hg/kg bw/day after treatment with thimerosal (I) and HgCl₂ [Hultman and Nielsen, 2001], respectively, in the drinking water. Systemic immune-complex deposits in the form of splenic vessel wall deposits showed a LOAEL of 147 (I) and 37 [Hultman and Nielsen, 2001] μ g Hg/kg bw/day for thimerosal and HgCl₂, respectively. The reason for the lower autoimmunogenic potency of thimerosal has not been studied. One explanation might be that the

autoimmunogen effect of thimerosal is caused by the metabolically formed Hg^{2+} , which comprises a maximum of 40% of the total renal Hg tissue concentration (and less in lymph nodes). Another possibility is that the initial immunosuppression caused by thimerosal (II) suppresses the autoimmunogen effect, and increases the threshold dose for induction of ANoA.

Acceleration of spontaneous and induced autoimmunity

The LOAEL for the accelerating/aggravating effect of long-term treatment with thimerosal on spontaneous systemic autoimmunity was in the order: splenic vessel wall deposits < renal vessel wall deposits = serum IgG1 < ANA = redistribution of glomerular immune deposits < serum IgE (IV) (Table 2). The LOAEL for splenic and renal vessel wall deposits was 9 and 18 $\mu\text{g Hg/kg bw/day}$, respectively, which is distinctly lower than the LOAEL for de novo induction of splenic vessel wall deposits by thimerosal in H-2^s mice (147 $\mu\text{g Hg/kg bw/day}$), and also lower than the LOAEL for de novo induction of splenic vessel walls deposits by HgCl_2 in susceptible mice (37 $\mu\text{g Hg/kg bw/day}$). It should be noted that since the splenic vessel wall deposits in the thimerosal-treated ZBWF1 mice were seen also in the lowest dose group used, the NOAEL could not be established and might be lower than the LOAEL observed. Accelerating/aggravating effects of Hg on spontaneous autoimmunity has usually been studied using higher doses than those discussed here. 420 $\mu\text{g Hg/kg bw/day}$ for 4 weeks to ZBWF1 mice caused an acceleration of ANA, lymphoproliferation, hyperimmuno-globulinemia, and renal and systemic vessel wall IC deposits in ZBWF1 mice [Pollard *et al.*, 1999]. In another study, 400 $\mu\text{g Hg/kg bw/day}$ to ZBWF1 mice for 4 weeks caused mesangial IgG (but not C3c) deposits, and vessel wall deposits were not mentioned [Abedi-Valugerdi *et al.*, 1997]. Lower doses of Hg have not used in the ZBWF1 model by others. However, in a study of the spontaneous, systemic autoimmune murine BXSB model [Pollard *et al.*, 2001], a statistically significant increase of ACA was found using a dose of 3.4 $\mu\text{g Hg/kg bw/day}$. In addition, 2 weeks treatment to host and donors from two strains not genetically susceptible to HgIA using 10 $\mu\text{g Hg/kg bw/day}$ as HgCl_2 , accelerated the induced lupus-like graft-versus-

host disease condition [Pollard *et al.*, 2001]. These observations show that organic and inorganic Hg has the ability to aggravate/accelerate manifestations of both a spontaneous (genetic) and induced type systemic autoimmunity at a dose of 3-10 µg Hg/kg bw/day. The dose threshold for inducing HgIA de novo ANoA is similar (11 µg Hg/kg bw/day), or slightly higher (37 µg Hg/kg bw/day) using HgCl₂ (III), [Hultman and Nielsen, 2001]. In contrast, the threshold for de novo induction of ANoA and systemic IC deposits by thimerosal is considerably higher, 118 and 147 µg Hg/kg bw/day.

What do these new observation add with regard to the risk for effects of thimerosal on humans? The serological effects including acceleration of ANA occurred at a dose level of at least 147 mg Hg/kg b.w/day, which is only seen in connection with accidental exposure. For example, the median daily dose of Hg caused by exposure to ethylmercury p-toluene sulfonanilide in bread from an outbreak of poisoning by dressed seeds was 180 µg/kg bw for 45 days, and infusion of plasma with erroneous high thimerosal concentration for 90 days was calculated to have resulted in a daily dose of 160 µg Hg/kg bw/day [Magos, 2001]. The main recent cause for thimerosal exposure in the human population is however as a preservative in vaccines. The maximum cumulative dose of mercury from thimerosal in vaccines before 1999 in the U.S. was estimated to 200 and 275 µg in a 6-month- and 2-year-old child [Stratton, 2001]. By using a cautious way of calculation, using an averaging period of only one day, a maximum single-day exposure of 10-15 µg Hg/kg bw is obtained [Stratton, 2001]. This is clearly within the dose range observed for the accelerating effect on development of systemic vessel wall IC deposits observed in the present study using the autoimmune-prone ZBWF1 hybrid mice. In addition to the requirement for autoimmune-prone genotype, the minimum exposure time needed in order to accelerate development of vessel wall deposits using low doses of thimerosal need to be further studied. However, a calculation of the “safe” dose (reference dose – EPA; allowable daily intake – WHO; minimal risk level – ATSDR) for the immunological effects of EtHg based on the present data from induction of splenic vessel wall IC deposits in ZBWF1 mice would be as follows. The observed LOAEL of 9 µg Hg/kg

bw/day has to be divided by uncertainty factors in order to obtain the reference dose [Barnes and Dourson, 1988; Clarkson, 1992]. An uncertainty factor of 10 applies when data from animals are extrapolated to humans. A second uncertainty factor of 10 has to be used since only the LOAEL and not the NOAEL is available. Finally, an uncertainty factor of 10 to compensate for variation in sensitivity among members of the human population might apply. The reference dose for IC-inducing effects of EtHg would therefore be $9 \mu\text{g Hg/kg bw/day}/1000 = 0.009 \mu\text{g Hg/kg bw/day}$. Since so little information is available on the toxicology of EtHg, the reference dose used by the regulatory agencies for EtHg is the dose for MeHg, which varies between 0.1 and 0.47 $\mu\text{g /kg bw/day}$ [NRS, 2000]. In 2000, the Committee on the toxicological effects of methylmercury of the National Research Council reexamined the question of a safe dose for MeHg exposure, which resulted in an acceptance of the reference dose established by the Environmental Protection Agency (EPA) of 0.1 $\mu\text{g/kg/day}$ [NRS, 2000]. The reference dose calculated from the study in the ZBWF1 mice is 11-fold lower than the reference dose accepted by EPA, which is founded on the risk for neurodevelopmental effects. However, the Committee concluded that while there are no data on the effect of MeHg on the human immune system [NRS, 2000], there is a reasonable possibility that significant effects on the immune system might develop at doses lower than those affecting neurodevelopment, a view further reinforced in a recent article [Dourson *et al.*, 2001]. It is therefore of interest that the present study does indicate effects on the immune system, in the form of vessel wall IC-deposits, in susceptible individuals at a dose significantly lower than the current RfD.

Exposure to thimerosal as a preservative in vaccines and as antiseptics may seem as a dispensable mercury load to the public, although it has been argued that the removal of thimerosal from vaccines would produce no more than 50% reduction of mercury exposure in infancy and <1% over a lifetime [Bigham and Copes, 2005]. Interestingly, the continued use of thimerosal as a preservative in the multi-dose vials used for vaccine delivery in poor countries has very recently been advocated [Clements and McIntyre, 2006], but should be viewed in light of the findings presented in this thesis.

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