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Dose–response study of thimerosal-induced murine systemic autoimmunity

S. Havarinasab,[a] L. Lambertsson,[b] J. Qvarnström,[b] and P. Hultman[a,*]

[a] *Molecular and Immunological Pathology (AIR), Department of Molecular and Clinical Medicine, Linköping University, SE-581 85 Linköping, Sweden*
[b] *Department of Chemistry, Analytical Chemistry, Umeå University, SE-901 87 Umeå, Sweden*

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Abstract

The organic compound ethylmercurithiosalicylate (thimerosal), which is primarily present in the tissues as ethylmercury, has caused illness and several deaths due to erroneous handling when used as a disinfectant or as a preservative in medical preparations. Lately, possible health effects of thimerosal in childhood vaccines have been much discussed. Thimerosal is a well-known sensitizing agent, although usually of no clinical relevance. In rare cases, thimerosal has caused systemic immune reactions including acrodynia. We have studied if thimerosal might induce the systemic autoimmune condition observed in genetically susceptible mice after exposure to inorganic mercury.

A.SW mice were exposed to 1.25–40 mg thimerosal/l drinking water for 70 days. Antinucleolar antibodies, targeting the 34-kDa protein fibrillarin, developed in a dose-related pattern and first appeared after 10 days in the two highest dose groups. The lowest observed adverse effect level (LOAEL) for antifibrillarin antibodies was 2.5 mg thimerosal/l, corresponding to an absorbed dose of 147 μg Hg/kg bw and a concentration of 21 and 1.9 μg Hg/g in the kidney and lymph nodes, respectively. The same LOAEL was found for tissue immune-complex deposits. The total serum concentration of IgE, IgG1, and IgG2a showed a significant dose-related increase in thimerosal-treated mice, with a LOAEL of 5 mg thimerosal/l for IgG1 and IgE, and 20 mg thimerosal/l for IgG2a. The polyclonal B-cell activation showed a significant dose–response relationship with a LOAEL of 10 mg thimerosal/l. Therefore, thimerosal induces in genetically susceptible mice a systemic autoimmune syndrome very similar to that seen after treatment with inorganic mercury, although a higher absorbed dose of Hg is needed using thimerosal. The autoimmune syndrome induced by thimerosal is different from the weaker and more restricted autoimmune reaction observed after treatment with an equipotent dose of methylmercury.

Keywords: Thimerosal; Ethylmercury; Mice; Autoimmunity

Introduction

In a recent review Clarkson (2002) described thimerosal in vaccines as one of three modern faces of mercury, the two other being methylmercury in fish and mercury vapor from dental amalgam fillings. Thimerosal is an organic, alkylmercury compound in which an organic radical, ethylmercury, is bound to the sulfur atom of the thiol group of salicylic acid. The type of anion attached to ethylmercury affects neither the distribution of mercury in the body nor the toxicity (Suzuki et al., 1973; Ulfvarson, 1962), while the organic radical has a strong impact on both (Magos, 2003). Ethylmercury and its decomposition product, Hg^{2+}, rapidly accumulate in the tissues (Magos, 2001).

Ethylmercury has been frequently used since it was first synthesized in the 19th century. When used as a seed disinfectant in developing countries, it caused several outbreaks of poisoning with neurological symptoms and signs similar to those of methylmercury intoxication (Clarkson, 2002). Such manifestations have also been recorded after occupational exposure and after use as a wound disinfectant and a preservative in medical preparations (Magos, 2001). A number of severe intoxications and deaths have occurred with the use of erroneous concentrations of thimerosal in medical preparations during the last 30 years (Axton, 1972; Suzuki et al., 1973).

Since the 1930s, thimerosal has been used world-wide as a preservative in vaccines, a use that was approved as late as 1976 by the U.S. Food and Drug Administration

* Corresponding author. Fax: +46-13132257.
E-mail address: perhu@imk.liu.se (P. Hultman).

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(Stratton et al., 2001). However, after introduction of more extensive childhood immunization schedules in the early 1990s, it was realized that 2-year-old children might be exposed for up to 275 μg of mercury, which caused concern for possible health effects, especially neurodevelopmental disorders, and led to restrictions in the use of thimerosal as a preservative in U.S. childhood vaccines (Ball et al., 2001).

Poisoning with ethyl- and methylmercury is characterized by an interval of several weeks between the first exposure and the onset of symptoms. This similarity is likely to be due to similar tissue distribution of the two compounds (Ball et al., 2001; Goldman and Shannon, 2001). However, ethylmercury induces significantly lower Hg concentration in the brain but higher concentration in the kidney compared with methylmercury (Suzuki et al., 1963). In addition, conversion to inorganic mercury is believed to be more rapid for ethylmercury, which leads to higher accumulation of inorganic mercury in the brain, blood, and kidneys after exposure to ethylmercury (Magos et al., 1985).

Mercury in the form of metallic mercury (vapor) and inorganic ionic as well as organic mercury (methylmercury) compounds may cause adverse effects in the human immune system, including local and systemic hypersensitivity (Eneström and Hultman, 1995; Pollard and Hultman, 1997). Thimerosal, with ethylmercury as the main sensitizing moiety (Santucci et al., 1998), is a frequent sensitizer according to the diagnostic patch test. In recent studies, the prevalence of a positive patch test was 8.7% (Suneja and Belsito, 2001), 11.8% (Wöhrl et al., 2003), and 13.4% (Gonçalo et al., 1996) in patients with suspected contact allergy. Sensitization is more common in individuals with certain polymorphisms in the glutathione transferase genes (Westphal et al., 2000). Although clinically relevant hypersensitivity to thimerosal is unusual (Suneja and Belsito, 2001; van't Veen and van Joost, 1994), thimerosal has in rare cases caused systemic hypersensitivity with manifestations in the skin (Zenarola et al., 1995) and the airways (Maibach, 1975), as well as acrodynia (Matheson et al., 1980). Acrodynia occurs in only 1:500 mercury-exposed children, indicating that the susceptibility is genetically regulated (Black, 1999).

Genetically regulated susceptibility is a characteristic also of mercury-induced autoimmunity (HgIA) in rodents (Fournié et al., 2001; Pollard and Hultman, 1997). The induction of antinuclear autoantibodies (ANA) by mercury in mice is highly specific and controlled in large part by the mouse MHC (H-2): mice with certain H-2 haplotypes develop autoantibodies to fibrillarin (AFA), a nucleolar protein, in response to mercury (Goter-Robinson et al., 1986; Hultman et al., 1992; Mirtcheva et al., 1989). The mercury-induced AFA target epitopes on fibrillarin that is also recognized by the AFA present in a subset of humans with systemic scleroderma (Takeuchi et al., 1995). The susceptible mouse strains develop systemic immune-complex deposits, which contain AFA (Hultman and Eneström, 1988). Other manifestations include lymphoproliferation and hypergammaglobulinemia (Pollard and Hultman, 1997).

Table 1
Autoimmune parameters in A.SW (H-2^s) mice treated with equipotent doses of mercury in the form of mercuric chloride or methylmercury

Immune parameter	$HgCl_2$	MeHg
Serum antinucleolar antibodies	+++[a]	+[b]
Polyclonal B-cell activation	++[a]	±0[b]
Total serum IgG1 concentration	+[a]	±0[b]
Total serum IgG2a concentration	+[a]	+[b]
Total serum IgE concentration	+++[a]	(+)[b]
Immune-complex deposits		
Glomerular deposits	++[c]	±0[b]
Systemic vessel walls deposits	++[c]	±0[b]

±0, no change; +, slight increase; ++, moderate increase; +++, strong increase.
[a] Johansson et al., 1998.
[b] Hultman and Hansson-Georgiadis, 1999.
[c] Hultman et al., 1996.

The ongoing debate on thimerosal in vaccines has revealed a lack of information on the toxicology of thimerosal, and conclusions were to a large extent arrived at by making an analogy with the toxicology of methylmercury (Stratton et al., 2001). However, equimolar doses of Hg in the form of methylmercury and inorganic mercury have different effects on the immune system (Table 1). We have therefore studied the effect of thimerosal on the immune system using a mouse model for induction of autoimmunity. Thimerosal induces an autoimmune reaction similar to that of inorganic mercury, which is in several aspects different from that of methylmercury (Hultman and Hansson-Georgiadis, 1999).

Material and methods

Animals. Female A.SW (H-2^s) mice were obtained from Taconic M&B, Ry, Denmark. All mice were 9–12 weeks of age at onset of the experiments. Mice were housed under 12:12-h dark/light cycles, kept in steel-wire cages, given pellets (Type R 70; Lactamin, Vadstena, Sweden) and tap water ad libitum. The pellets were examined by gas chromatography–inductively coupled plasma–mass spectrometry (Qvarnström et al., 2003) and contained 23 ng Hg^{2+}/g and 4 ng methylmercury as Hg/g, whereas the ethylmercury concentration was below the detection limit (0.1 ng/g).

Thimerosal treatment. Ethylmercurithiosalicylate, $C_9H_9HgNaO_2S$, thimerosal (Fluka, Seelze, Germany), contained less than 1% free Hg (Qvarnström, unpublished observations) as assessed by gas chromatography–inductively coupled plasma–mass spectrometry (Qvarnström et al., 2003). Groups of five to eight female A.SW received drinking water ad libitum with thimerosal for 70 days using the following concentrations: 1.25, 2.5, 5, 10, 20, and 40 mg/l. Whereas

the actual consumption of drinking water was not monitored in this study, a previous study using mercuric chloride (Hultman and Nielsen, 1998) showed no significant differences in drinking water consumption over a wide dose range (1–16 mg $HgCl_2$/l). Furthermore, in the present study, a highly significant dose–response relationship was observed between the concentration of thimerosal in the drinking water and the body burden assessed by the renal mercury concentration (see below). Drinking water was freshly prepared weekly using tap water and thimerosal. Controls received tap water without any additions.

Blood and tissue sampling. Blood was obtained after 10, 42, and 70 days of treatment in one set of animals. In a second set of animals, blood was collected after 21 days, and pieces of the liver, spleen, and right kidney were obtained after 42 days for examination of immune deposits and histology. The mesenterial lymph nodes and the left kidney were excised using instruments regularly washed in a fresh solution of 65% HNO_3, weighed, and stored in Eppendorf tubes at −70 °C before analyzed for the total concentration of Hg.

Serum antinuclear antibodies by indirect immunofluorescence. Antibodies to nuclear antigens (ANA) were detected by indirect immunofluorescence (IIF) as previously described (Hultman et al., 1989). HEp-2 slides (Binding Site Ltd., Birmingham, England) were used as the antigenic substrate, serum diluted 1:40 for screening purposes, and bound antinuclear antibodies detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibodies (Sigma, St Louis, Missouri, USA) diluted 1:50. Sera showing a positive nuclear staining at a dilution of 1:40 were further assessed by diluting twofold up to 1:81 920. The pattern and titer of the serum antinuclear antibodies was recorded.

Serum antinuclear antibodies assessed by immunoblotting. 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 (Chan and Pollard, 1992), aliquots of boiled nucleoli were SDS-PAGE separated using a 12.5% gel, and electrophoretic transfer to 0.45-μm nitrocellulose membranes (BioRad Lab, Hercules, CA, USA) was performed for 1 h at 0.8 mA/cm^2 under water cooling (Criterion Blotter; BioRad Lab). Nitrocellulose strips were blocked in a Tris-buffered solution (TBS)–5% nonfat dry milk (blotting grade; BioRad Lab)–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, Birmingham, AL, USA) diluted 1:5000, followed by enhanced chemiluminescence (ECL Western blotting detection reagents; Amersham, Stockholm, Sweden).

Serum antichromatin antibodies assessed by ELISA. Antichromatin antibodies (ACA) were measured using the method of Burlingame and Rubin (1990). Calf thymus chromatin (180 μl/well) in distilled water was added to ELISA plates (Nunc, Copenhagen, Denmark) followed by 20 μl of 10 × PBS. After overnight incubation at 4 °C, the plates were post-coated with gelatin, incubated with serum, washed, alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG antibody (Caltag Laboratories, Burlingame, CA, USA) added, followed by washing and addition of substrate. The optical density was read at 405 nm, and background values were subtracted.

Serum anti-ssDNA antibodies assessed by ELISA. The method used has been described before (Johansson et al., 1997). Microtiter plates (Nunc) were coated overnight with 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 washed six times with BSA–Tween–PBS, and incubated with ALP-conjugated rabbit anti-mouse Ig (reacting with IgG, IgM, and IgA) (Sigma) diluted in BSA–Tween–PBS. The plates were repeatedly washed, substrate added, the reaction stopped with 3 M NaOH, the optical density measured at 405 nm, and the background subtracted. A pool of sera from MRL-*lpr/lpr* mice was used as positive control. Using a monoclonal antibody (clone HB2) reacting with dsDNA (Sera-Lab), we detected no contamination with dsDNA in the coating (data not shown).

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

Serum IgM, IgG1, and IgG2a concentrations assessed by ELISA. Microtiter plates (Nunc) were coated overnight at 4 °C with rat anti-mouse IgG1 and IgM MAb (LO-IMEX, Brussels, Belgium). The plates were washed, blocked with PBS, and the wells incubated with diluted serum. Bound IgG1 and IgM were detected using HRP-conjugated rat anti-mouse IgG1 and rat anti-mouse IgM MAb (LO-IMEX), respectively. After washing and addition of substrate, the optical density in the wells was measured at 450 nm, and the background values in wells coated with PBS instead of serum were subtracted. Standard curves using mouse myeloma proteins of the IgG1 and IgM isotype (LO-IMEX)

were used to obtain the actual concentration. For serum IgG2a analysis, microtiter plates (Nunc) were coated with purified anti-mouse Ig κ-light Chain (Pharmingen Inc., San Diego, CA, USA), the wells were incubated with diluted sera, and bound IgG2a was detected with ALP-conjugated anti-mouse IgG2a (Pharmingen). The IgG2a concentration in the samples was obtained from a standard curve using purified IgG2a (Pharmingen).

Serum IgE concentration assessed by ELISA. Serum IgE was determined as described before (Warfvinge et al., 1995). Briefly, microtiter plates were coated with rat anti-mouse IgE (Southern Biotechnology), followed by blocking and incubation with diluted serum. Bound IgE was detected by HRP-conjugated goat anti-mouse IgE (Nordic Immunological Lab, Tilburg, Netherlands), and the IgE concentration in the samples was derived from a standard curve using mouse myeloma protein of the IgE isotype (Sigma).

Tissue immune deposits. Pieces of the right kidney were examined with direct immunofluorescence as described before (Hultman et al., 1995) using FITC-conjugated goat anti-mouse IgG and IgM (Sigma), as well as anti-C3c antibodies (Organon-Technica, West Chester, PA, USA). The titer of glomerular IgG and C3c deposits was determined by serial dilution of the antibodies to 1:5120. Pieces of the spleen were examined using anti-IgG and anti-C3c abs diluted 1:40. The presence of IgG and C3c deposits in the renal and splenic vessel walls was recorded.

Determination of total mercury concentration in the kidneys and the mesenterial lymph nodes. The frozen tissues were thawed and cut with a scalpel into 5- to 10-mg pieces that were directly analyzed in a Leca AMA 254 mercury analyzer.

Statistical methods. Statistical analyses were performed using GraphPad Software Inc. The Spearman rank correlation test was used to study dose–response relationships. Concerning differences between controls and thimerosal-treated mice, most parameters were analyzed by the non-parametric Kruskal–Wallis test followed by Dunn's post test. Differences in the frequency of tissue vessel immune-complex deposits was however analyzed by Fisher's Exact Test. Results were considered statistically significant at a level of $P < 0.05$.

Results

Animal health

Two of the mice given 40 mg thimerosal/l died in connection with blood sampling after 42 days, and one mouse died of unknown cause after 8 weeks. These deaths indicate an increased vulnerability in the highest dose group especially during stress such as blood sampling. However, the behavior in all thimerosal-treated mice was normal, and regular examinations revealed no signs of disease. Since LD50 for oral exposure to thimerosal is 91 mg/kg bw (National Toxicology Program, 2003), and 40 mg thimerosal/l drinking water corresponds to a daily dose of thimerosal of 4.8 mg/kg bw, this was not unexpected.

Development of serum antinuclear antibodies

Sufficiently high doses of thimerosal induced antinucleolar antibodies (ANoA), which stained the nucleoli in a "clumpy" pattern, gave a modest staining of condensed chromosomes and stained multiple dots in the nucleus, a pattern compatible with antifibrillarin antibodies (Pollard and Hultman, 1997). The two highest concentrations of thimerosal (20 and 40 mg/l) induced ANoA in some mice already after 10 days administration (Fig. 1A). After 42 days of administration, all mice receiving 10 mg/l or more of thimerosal showed high titers of ANoA, although the response was heterogeneous in mice given 2.5 and 5.0 mg/l (Fig. 1B). In the 2.5 mg/l group, two mice showed a high titer after 42 days, although the other five mice remained negative also after 70 days of treatment (Fig. 1C). In the 5 mg/l group, four mice showed modest-high titers of ANoA after 42 days of treatment. After 70 days of treatment, another mouse had developed ANoA, although two mice remained negative. A single mouse treated with 1.25 mg thimerosal/l showed ANoA after 42 and 70 days.

The correlation between the dose of thimerosal and the reciprocal ANoA titer was significant after 42 and 70 days ($P = 0.0004$ and 0.0238, respectively; Spearman rank correlation test), but the ANoA titer plateaued after 42 and 70 days of treatment in mice given 10–40 mg thimerosal/l. Sera from a few controls, as well as thimerosal-treated mice, showed ANA with another pattern than nucleolar, but none of the pattern showed any correlation with thimerosal treatment (data not shown).

Specificity of the antinucleolar antibodies

Immunoblotting using mouse liver nucleoli as antigen showed that ANoA-positive sera with a titer of 1:320 or more generally reacted with a 34-kDa protein that was also targeted by a human reference serum against fibrillarin (Fig. 2). A few sera also reacted with nucleolar proteins of an apparent molecular weight of 65–70 kDa (Fig. 2) as described before (Pollard and Hultman, 1997). The blotting reaction was generally stronger using sera with a higher ANoA titer. The two mice in the 2.5 mg/l group with ANoA both showed a modest reactivity with the 34-kDa protein in immunoblotting (Fig. 2, lane 7, and data not shown). A single mouse given 1.25 mg thimerosal/l showed an ANoA titer of 1:5120 after 42 and 70 days. However, repeated immunoblotting of these two sera from this mouse did not demonstrate any convincing reactivity with nucleolar pro-

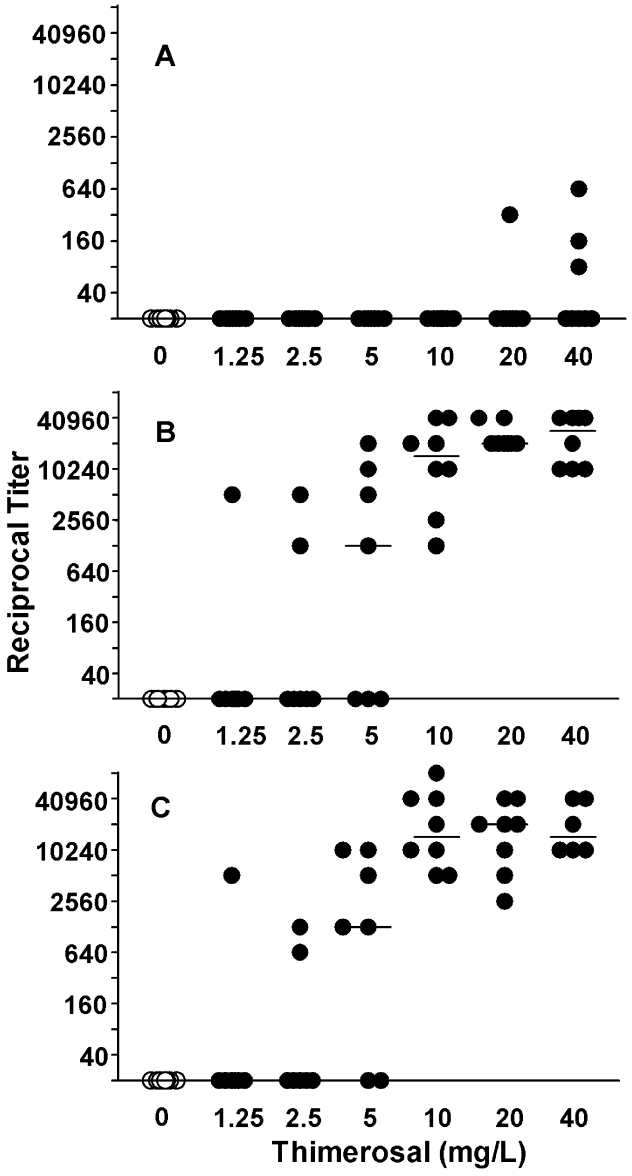

Fig. 1. Reciprocal titer of serum IgG antinucleolar antibodies in control mice (0) and in mice treated with 1.25–40 mg thimerosal/l drinking water for 10 (A), 42 (B), or 70 (C) days as determined by indirect immunofluorescence using HEp-2 cells as a substrate. Horizontal scale bars denote median titer.

teins (data not shown). Due to the high titer of ANoA, AFA should have been readily detectable by immunoblotting. We therefore conclude that this mouse did not develop AFA. Sera from the controls showed no reaction with mouse liver nucleoli proteins.

Serum antichromatin antibodies

The serum ACA level was significantly correlated with the dose of thimerosal after 42 and 70 days ($P = 0.0028$ and $P = 0.024$, respectively). The increase in ACA was statistically significant ($P < 0.01$) after 10 days in mice given 40 mg thimerosal/l group compared with the controls. After 42 days, mice given 5 mg thimerosal/l or more showed a significant increase of ACA compared with controls. All concentrations of thimerosal, except 5 mg/l, caused a significant ($P < 0.05$) increase in ACA after 70 days compared with the controls. However, the absolute increase in ACA was modest. While the controls generally showed ACA levels of 0.015–0.065, measured as the optical density at 405 nm, the thimerosal-treated mice did not attain a higher ACA value than 0.1 (data not shown).

Serum immunoglobulins

The serum IgE concentration showed a significant correlation with the dose after 10 days ($P = 0.0238$). The mean serum IgE concentration increased 36- to 39-fold after 10 days of treatment with 10–40 mg thimerosal/l compared with the controls. In the 5 mg/l group, the increase was eightfold (Fig. 3). After 42 and 70 days, the mean serum IgE concentration was not increased more than twofold at any dose of thimerosal as compared with the controls, although a few thimerosal-treated mice showed up to an eightfold increase. After 10 days of treatment with a dose of 5–40 mg thimerosal/l, serum IgE was significantly increased compared with the controls. Serum IgG1 showed a dose-related

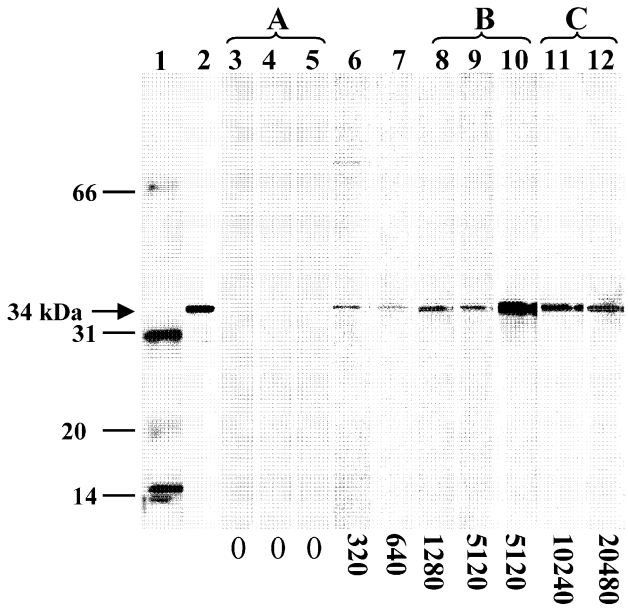

Fig. 2. Immunoblotting of sera from A.SW mice using SDS-PAGE separated mouse liver nucleoli. Lane 1: molecular weight markers (kDa). Lane 2: human reference serum blotting the 34-kDa protein fibrillarin. Block A: sera from three control mice. Lane 6: serum from a mouse treated with 20 mg thimerosal/l drinking water for 10 days. Lane 7: serum from a mouse treated with 2.5 mg thimerosal/l for 70 days. Block B: mice treated with 5 mg thimerosal/l for 42 (lane 9) or 70 (lane 8 and 10) days. Block C: sera from mice treated with 10 mg thimerosal/l for 42 days. Numerals beneath the figure are the reciprocal titer of ANoA. All ANoA-positive sera targeted a protein with an apparent molecular weight of 34-kDa corresponding to fibrillarin. A single serum reacted in addition with a protein of 65–70 kDa (lane 6).

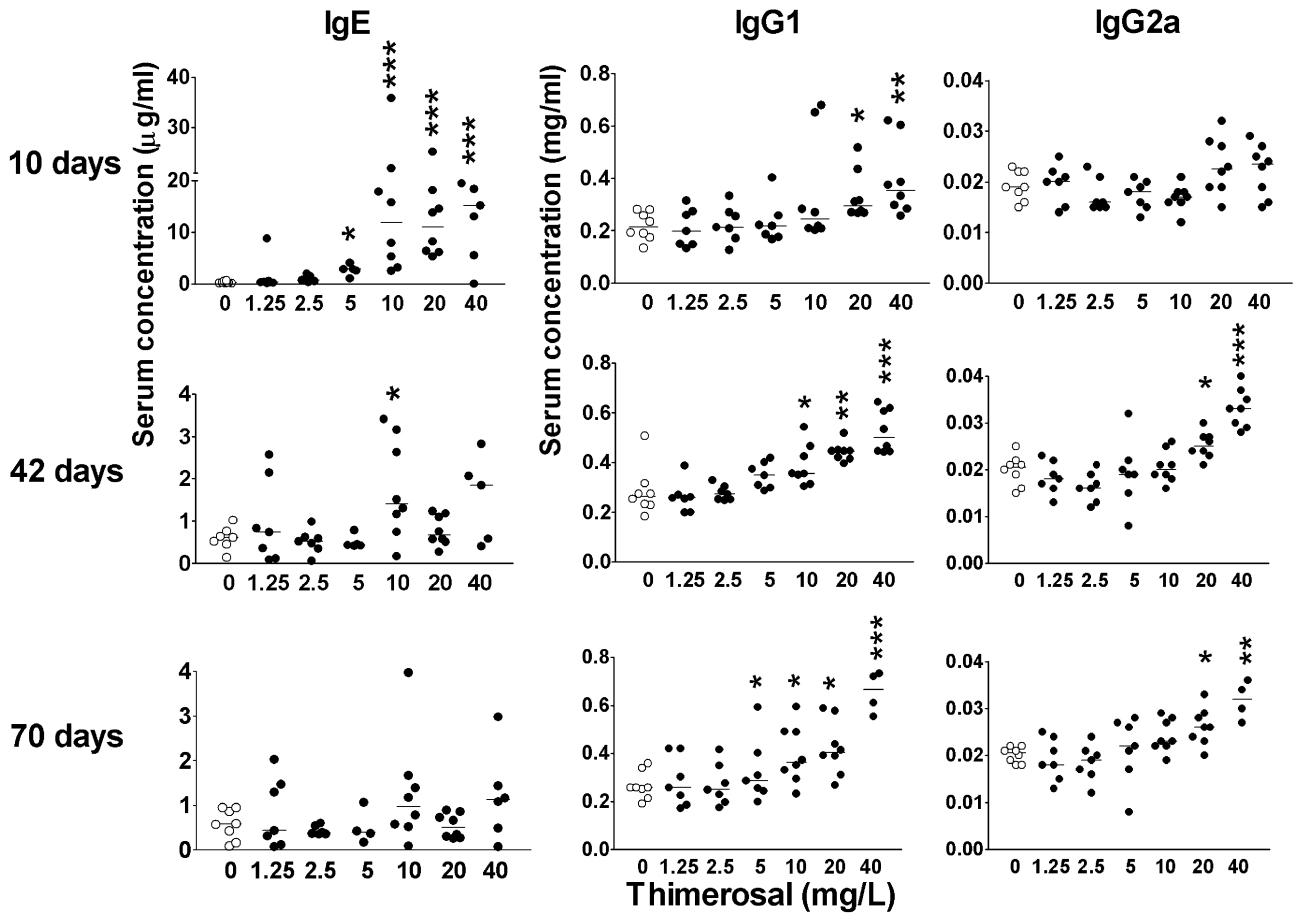

Fig. 3. Serum concentrations of IgE, IgG1, and IgG2a in mice after 10, 42, and 70 days of treatment with 1.25–40 mg thimerosal/l drinking water or controls (0). Horizontal scale bars denote median value. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the controls (Kruskal–Wallis followed by Dunn's post test).

increase after 42 and 70 days ($P = 0.0123$ and 0.0028, respectively). Serum IgG1 was significantly increased compared with the controls in the groups receiving 20 and 40 mg thimerosal/l after 10 days, in the 10–40 mg/l groups after 42 days, and in the 5–40 mg/l groups after 70 days (Fig. 3). Serum IgG2a was significantly increased in the 20–40 mg/l groups after 42 and 70 days compared with the controls, and there was a significant dose–response relationship after 70 days ($P = 0.0238$).

Polyclonal B-cell activation markers

The total (IgA, IgG, IgM) anti-ssDNA ab level showed a significant dose-related increase after 10, 42, and 70 days ($P = 0.012$, 0.00040, and 0.0028, respectively) and was significantly increased compared with the controls in the 10–40 mg/l groups after 10, 42, and 70 days. In addition, the 2.5 mg/l group (but not the 5 mg/l group) showed a significant increase after 70 days (Fig. 4). The total anti-DNP ab level showed a significant dose-related increase after 10, 42, and 70 days ($P = 0.0028$, 0.048, and 0.048, respectively) and was significantly increased compared with the controls in the 10–40 mg/l group s after 10 days, in the 10 and 40 mg/l groups after 42 days, and in the 20–40 mg/l groups after 70 days (Fig. 4). The serum IgM concentration showed a significant dose-related increase after 10, 42, and 70 days ($P = 0.0067$, 0.012, and 0.048, respectively) and was significantly increased compared with the controls in the 2.5 and 10–40 mg/l groups after 10, 42, and 70 days (Fig. 4). Taken together, the data show a significant dose-related increase in these parameters for assessment of polyclonal B-cell activation (Izui et al., 1977). The lowest dose of thimerosal that caused a significant increase in all three parameters for polyclonal B-cell activation compared with the controls was 10 mg/l, which is therefore considered to be the lowest observed adverse effect level (LOAEL) for polyclonal B-cell activation.

Tissue immune-complex deposits

The titer of granular IgG deposits in the renal mesangium showed a correlation with the dose ($P < 0.0001$), and the titer reached a plateau level at a dose of 20 mg thimerosal/l (Table 2). The mean titer of C3c in the mesangium showed

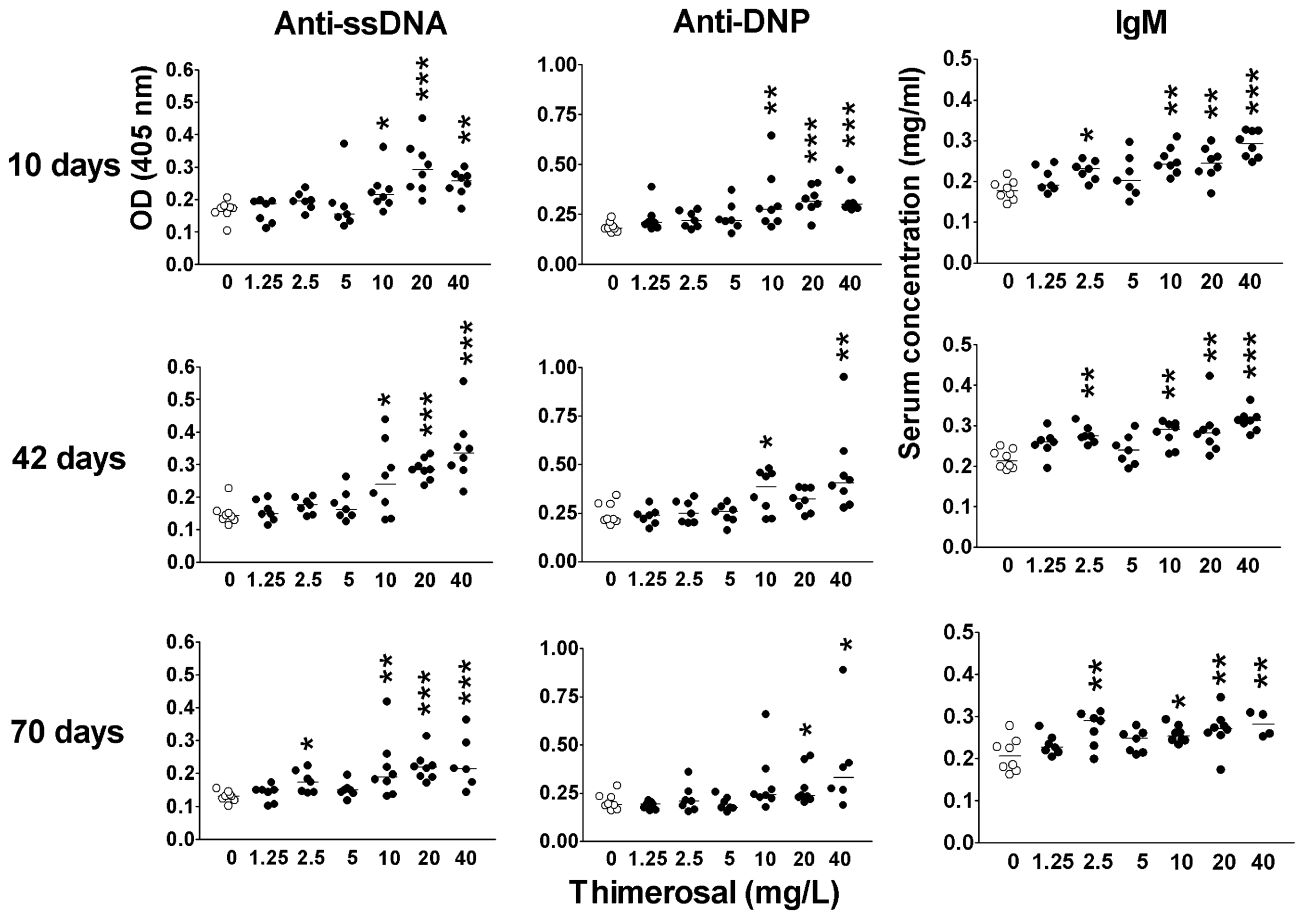

Fig. 4. The polyclonal B-cell activation markers anti-ssDNA and anti-DNP antibodies, and serum IgM, after 10, 42, and 70 days of treatment with 1.25–40 mg thimerosal/l drinking water or controls (0). Horizontal scale bars denote median value. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the controls (Kruskal–Wallis followed by Dunn's post test).

a correlation with the dose ($P < 0.0001$) and plateaued at a dose of 5 mg/l. The graded titer of mesangial IgM deposits showed a significant ($P = 0.0059$) correlation with the dose, and the mean graded titer was increased compared with the controls at a dose of 5 mg thimerosal/l and higher. Granular renal and splenic vessel wall deposits of IgG developed in a significant ($P < 0.0001$) correlation with the dose, first appeared in a fraction of mice given a dose of 2.5 mg/l, but was present in all mice given at least 10 mg thimerosal/l. Granular deposits of C3c in the vessel walls first appeared at

Table 2
Tissue immune-complex deposits in thimerosal-treated and control mice

Treatment/ dose (mg/l)	No.	Immune-complex deposits						
		Renal mesangium			Vessel walls			
		IgG[a]	C3c[a]	IgM[b]	Kidney		Spleen	
					IgG (% positive)	C3c	IgG	C3c
Controls	5	0	224 ± 39	1.4 ± 0.24	0	0	0	0
1.25	5	0	512 ± 74	1.2 ± 0.20	0	0	0	0
2.5	5	512 ± 78	928 ± 229	1.2 ± 0.20	40	0	80*	0
5.0	5	704 ± 256	1408 ± 313	1.8 ± 0.20	80*	20	80*	20
10	5	768 ± 128	832 ± 192	1.8 ± 0.20	100*	40	100*	20
20	5	1152 ± 128**	1344 ± 356	1.8 ± 0.20	100*	100*	100*	80*
40	2	1280 ± 0	1920 ± 640	2.0 ± 0	100*	50	100*	100*

[a] Mean reciprocal titer ± SEM.
[b] Grading, 0–3: figures denote mean ± SEM.
* Significantly different from controls $P < 0.05$; Fisher's Exact Test.
** Significantly different from controls $P < 0.01$; Kruskal–Wallis and Dunn's post test.

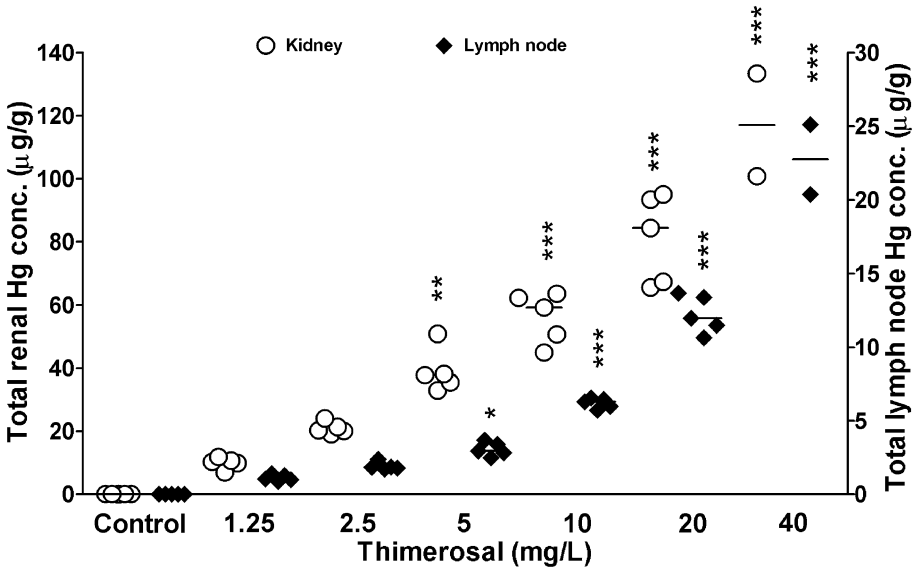

Fig. 5. The total mercury concentration in kidney and mesenterial lymph node after 42 days of treatment with 1.25–40 mg thimerosal/l drinking water or controls (0). Horizontal scale bars denote median values. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the controls (Kruskal–Wallis followed by Dunn's post test).

a dose of 5 mg/l, but a dose of 20–40 mg/l was needed in order for deposits to develop in all treated mice. The vessel wall C3 deposits showed a significant ($P < 0.0001$) correlation with the dose.

Tissue mercury concentrations

Controls contained 0.05–0.08 μg Hg/g renal tissue and ca. 0.001 μg Hg/g lymph node tissue. The total mercury concentration in both the kidney and the lymph nodes showed a significant correlation with the dose ($P = 0.00040$ in both tissues), and the mercury concentration was significantly higher in both the kidney and the mesenterial lymph nodes in mice given a dose of 5.0 mg thimerosal/l or more as compared with the controls (Fig. 5).

Discussion

We show in this study that genetically susceptible mice exposed to the organic ethylmercury compound thimerosal develop, in a dose-dependent pattern, all the features described in the systemic autoimmune condition occurring in genetically susceptible mice after exposure to inorganic mercury (Pollard and Hultman, 1997), whether given as metallic mercury vapor (Warfvinge et al., 1995) or mercuric chloride via the oral (Hultman and Eneström, 1992) or subcutaneous (Hultman and Eneström, 1989) route. This is in contrast to another organic mercury compound, methylmercury, which induces only modest titers of antifibrillarin autoantibodies, and none of the two other hallmarks of mercury-induced autoimmunity, a marked increase of IgE and systemic immune-complex deposits (Hultman and Hansson-Georgiadis, 1999). This difference between ethyl- and methylmercury is interesting because it has been argued that the toxicology of ethylmercury is similar to that of methylmercury due to similarities in chemistry and toxicokinetics between the two compounds (Ball et al., 2001; Goldman and Shannon, 2001).

Might there be other explanations for our findings than a qualitatively different effect on the immune system of ethyl- as compared with methylmercury? One possibility would be differences in the absorbed dose of Hg. The increase of IgE and induction of immune-complex deposits were first observed at a dose of 5.0 mg thimerosal/l drinking water. Given a mercury content of 49.6% in thimerosal, a drinking water consumption of 2.5 ml/day (Hultman and Nielsen, 1998), and an uptake of 95% in the gastrointestinal tract (Goldman and Shannon, 2001), the daily absorption using a dose of 5.0 mg/l would be 5.9 μg Hg/mouse, corresponding to 295 μg Hg/kg bw per day. In the methylmercury study, where genetically susceptible A.SW mice were also used (Hultman and Hansson-Georgiadis, 1999), treatment consisted of subcutaneous injections of 1.0 mg CH_3HgCl/kg bw every third day for 4 weeks. Given a mercury content of 80% in CH_3HgCl, the absorbed dose corresponds to 270 μg Hg/kg bw per day. Therefore, the absorbed dose of methylmercury in the previous study (Hultman and Hansson-Georgiadis, 1999) should have caused an increase of serum IgE and tissue immune-complex deposits if methylmercury had been equipotent with ethylmercury.

Another possible explanation for the different effects of ethyl- and methylmercury on the immune system would be different toxicokinetics. The pattern of tissue disposition of ethyl- and methylmercury is qualitatively similar (Clarkson, 2002). However, in a short-term study in mice (Suzuki

et al., 1963), using an equipotent dose of Hg given as ethyl- or methylmercury, ethylmercury caused higher levels of Hg in the kidney and the liver, but lower levels in the brain. A higher concentration of Hg in the kidney after ethyl- as compared with methylmercury exposure was also reported in a long-term study in rats, while the Hg concentration in the liver was similar (Ulfvarson, 1962). Studies on the accumulation of ethyl- and methylmercury in the immune system are scarce, but the short-term study in mice (Suzuki et al., 1963) showed similar accumulation of Hg in the spleen after exposure to the two compounds. A more rapid conversion of ethylmercury to inorganic mercury, as compared with methylmercury, has been demonstrated (Magos et al., 1985; Matheson et al., 1980). This would lead to an earlier and stronger effect on the immune system from ethylmercury exposure due to the potent interaction of inorganic mercury with the immune system. AFA and maximum levels of serum IgE are present already after 10 days of exposure to ethylmercury (present study). The crucial effects of mercury on the immune system, which induce autoimmunity, must therefore take place already during the first week of exposure. Preliminary studies have shown that Hg^{2+} make up 27–33% and 13–18% of the total Hg concentration in kidney and lymph nodes after 6 days of exposure to thimerosal (Qvarnström et al., 2003) and methylmercury (Hultman et al., unpublished observations), respectively. Therefore, the available data do not support differences in toxicokinetics as a likely explanation for the different effects observed on the immune system after exposure to ethyl- and methylmercury.

With regard to the dose of ethylmercury needed to induce autoimmunity in genetically susceptible mice, the LOAEL for AFA was 2.5 mg thimerosal/l drinking water, corresponding to an absorbed dose of 147 μg Hg/kg bw and 21 μg Hg/g kidney tissue. The LOAEL for ANoA/AFA after oral $HgCl_2$ exposure in female A.SW mice was in two different studies 0.5 and 1.25 mg $HgCl_2$/l drinking water, which corresponds to a daily absorbed dose of 11 and 28 μg Hg/kg bw and a renal mercury concentration of 0.71 and 2.4 μg Hg/g, respectively (Hultman and Enestrom, 1992; Hultman and Nielsen, 2001). The LOAEL for vessel wall immune-complex deposits in thimerosal-treated mice was the same as for induction of AFA (present study), which should be compared with 28 and 56 μg Hg/kg bw per day, corresponding to 2.4 and 3.8 μg Hg/g kidney tissue, respectively, after oral $HgCl_2$-exposure (Hultman and Enestrom, 1992; Hultman and Nielsen, 2001). Consequently, 5–13 and 3–5 times higher absorbed dose of Hg is needed to induce AFA and immune-complex deposits, respectively, after exposure to ethylmercury as compared with inorganic mercury. Considering that part of the ethylmercury in the tissues is converted to the immunologically more active inorganic mercury (Magos et al., 1985; Qvarnström et al., 2003), ethylmercury must have a considerably weaker autoimmune effect than inorganic mercury.

What about the risk for development of autoimmunity in humans due to ethylmercury exposure? Such an event requires two conditions. First, the individual should be genetically susceptible. Secondly, a sufficient exposure must take place. With regard to genetical susceptibility to mercury-induced autoimmunity in humans, there is a limited number of case reports of mercury-induced immune-mediated disease (Enestrom and Hultman, 1995), and Kazantzis et al. (1962) observed that only a few of similarly mercury-exposed persons developed adverse immunological reactions. Unfortunately, no biomarker exists for genetical susceptibility to adverse immune reactions after mercury exposure, but a recent review indicated that in all events, less than 1% of the population is at risk of being susceptible (Berlin, 2003).

Using the dose–response data in mice (present study), genetically susceptible humans would need to absorb at least 147 μg Hg/kg bw per day for at least 5 days to develop autoimmunity. Interestingly, 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 (Magos, 2001). Several cases with acute neuro- and nephrotoxicity have been described after doses of thimerosal ranging from 3 mg/kg bw to several hundred mg/kg bw (Ball et al., 2001). However, the main cause for thimerosal exposure in the human population in recent time is as a preservative in vaccines (Clarkson, 2002). The maximum cumulative dose of mercury from thimerosal in vaccines before late 1999 in the U.S. was estimated to 200 and 275 μg in a 6-month- and 2-year-old child, respectively (Stratton et al., 2001). Since mercury exposure due to vaccination is episodic, the time over which averaging of the exposure is performed becomes critical. The most cautious calculation used an averaging period of only 1 day, which would give rise to a maximum single-day exposure to mercury of 15–20 μg/kg bw (Stratton et al., 2001). This is approximately 10–15% of the daily dose that would need to be present for at least 5 days to induce autoimmunity in genetically susceptible humans (see above). We therefore conclude that there exists no significant risk for de novo induction of systemic autoimmunity in humans due to thimerosal in vaccines.

Autoimmune diseases occur with different frequencies in females and males, the female/male ratio being 7 for systemic lupus erythematosus and as high as 15 for Sjögren syndrome (Jacobson et al., 1997). Our study included only female mice, and our data are therefore formally not applicable to the male gender. However, because genetically susceptible male mice develop autoimmunity after exposure to the ultimate metabolite of thimerosal, inorganic mercury (Nielsen and Hultman, 2002), it can be assumed that males from genetically susceptible strains will develop autoimmunity after exposure to ethylmercury. After an equivalent

dose of mercuric chloride, male mice accumulate slightly more mercury than females (Nielsen, 1992). If the influence of gender differences in kinetics is excluded by using instead the absorbed and retained whole-body mercury deposition, females show a lower threshold for induction of AFA (Nielsen and Hultman, 2002). Furthermore, female mice need a lower threshold to reach 100% AFA response (Nielsen and Hultman, 2002). Female mice therefore have a higher sensitivity and responsitivity for induction of autoimmunity by inorganic mercury. With regard to the kinetics of mercury in humans, one study found no difference in the body burden between the genders as estimated by the total renal mercury concentration (Drasch et al., 1992), but another study showed significantly lower burden in males (Barregård et al., 1999). There exists no specific data for ethylmercury in humans, but it seems unlikely that males should absorb more mercury or be at higher risk for autoimmune manifestations than females.

However, recent discussions regarding the autoimmune effect of mercury are not only, or even mainly, concerned with the risk of inducing de novo autoimmune condition, but further the possibility that mercury might accelerate or aggravate spontaneously occurring systemic autoimmune conditions. These concerns have been derived from the finding of such effects in a number lupus-like mouse models (Al-Balaghi et al., 1996; Pollard et al., 1999, 2001). In one of these studies, the daily dose of Hg, administered as $HgCl_2$, which was needed to accelerate the autoantibody production in the spontaneous lupus-like condition of female BXSB mice, was 4 μg/kg bw (Pollard et al., 2001), which should be compared with the 11–28 μg Hg/kg bw needed for de novo induction of autoimmunity. Further studies are necessary to examine if and at what doses thimerosal may accelerate the manifestations of spontaneously occurring systemic autoimmunity.

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References

Al-Balaghi, S., Möller, E., Möller, G., Abed-Valugerdi, M., 1996. Mercury induces polyclonal B-cell activation, autoantibody production and renal immune complex deposits in young (NZB × NZW)F1 hybrids. Eur. J. Immunol. 26, 1519–1526.

Axton, J., 1972. Six cases of poisoning after a parenteral organic mercury compound (Merthiolate). Postgrad. Med. J. 48, 962–964.

Ball, L.K., Ball, R., Pratt, R.D., 2001. An assessment of thimerosal use in childhood vaccines. Pediatrics 107, 1147–1154.

Barregård, L., Svalander, C., Schütz, A., Westberg, G., Sällsten, G., Blohmé, I., Mölne, J., Attman, P.-O., Haglind, P., 1999. Cadmium, mercury and lead in kidney cortex of the general Swedish population: a study of biopsies from living kidney donors. Environ. Health Perspect. 107, 867–871.

Berlin, M., 2003. Mercury in Dental-Filling Materials—An Updated Risk Analysis in Environmental Medical Terms. The Swedish Governmental Dental Care Commission, Stockholm, pp. 23–24. www.dentalmaterial.gov.se.

Black, J., 1999. The puzzle of pink disease. J. R. Soc. Med. 92, 478–491.

Burlingame, R.W., Rubin, R.L., 1990. Subnucleosome structure as substrates in enzyme-linked immunosorbent assays. J. Immunol. Methods 134, 187–199.

Chan, E.K.L., Pollard, K.M., 1992. Autoantibodies to ribonucleoprotein particles by immunoblotting. In: Rose, N.R., Freidman, H., Fahey, J.L. (Eds.), Manual of Clinical Laboratory Immunology. American Society for Microbiology, Washington, D.C., pp. 755–761.

Clarkson, T.W., 2002. The three modern faces of mercury. Environ. Health Perspect. 110 (Suppl. 1), 11–23.

Drasch, G., Schupp, I., Riedl, G., Günther, G., 1992. Einfluss von amalgamfüllungen auf die quecksilberkonzentration in menschlichen organen. Dtsch. Zahnaerztl. Z. 47, 490–496.

Eneström, S., Hultman, P., 1995. Does amalgam affect the immune system? A controversial issue. Int. Arch. Allergy Immunol. 106, 180–203.

Fournié, G., Mas, M., Cautain, B., Savignac, M., Subra, J.-F., Pelletier, L., Saoudi, A., Lagrange, D., Calise, M., Druet, P., 2001. Induction of autoimmunity through bystander effects. Lessons from immunological disorders induced by heavy metals. J. Autoimmun. 16, 319–326.

Goldman, L., Shannon, M., 2001. Technical report: mercury in the environment: implications for pediatricians. Pediatrics 108, 197–205.

Gonçalo, M., Figueiredo, A., Gonçalo, S., 1996. Hypersensitivity to thimerosal: the sensitizing moiety. Contact Dermatitis 34, 201–203.

Goter-Robinson, C.J., Balazs, T., Egorov, I.K., 1986. Mercuric chloride-, gold sodium thiomalate-, and penicillamine-induced antinuclear antibodies in mice. Toxicol. Appl. Pharmacol. 86, 159–169.

Hultman, P., Eneström, S., 1988. Mercury induced antinuclear antibodies in mice: characterization and correlation with renal immune complex deposits. Clin. Exp. Immunol. 71, 269–274.

Hultman, P., Eneström, S., 1989. Murine mercury-induced immune-complex disease: effect of cyclophosphamide treatment and importance of T-cells. Br. J. Exp. Pathol. 70, 227–236.

Hultman, P., Eneström, S., 1992. Dose–response studies in murine mercury-induced autoimmunity and immune-complex disease. Toxicol. Appl. Pharmacol. 113, 199–208.

Hultman, P., Hansson-Georgiadis, H., 1999. Methyl-mercury induced autoimmunity in mice. Toxicol. Appl. Pharmacol. 154, 203–211.

Hultman, P., Nielsen, J., 2001. The effect of dose, gender and non-H-2 genes in murine mercury-induced autoimmunity. J. Autoimmun. 17, 27–37.

Hultman, P., Nielsen, J.B., 1998. The effect of toxicokinetics on murine mercury-induced autoimmunity. Environ. Health Perspect. 77, 141–148.

Hultman, P., Eneström, S., Skogh, T., 1989. Circulating and tissue immune complexes in mercury-treated mice. J. Clin. Lab. Immunol. 29, 175–183.

Hultman, P., Bell, L.J., Eneström, S., Pollard, K.M., 1992. Murine susceptibility to mercury: I. Autoantibody profiles and systemic immune deposits in inbred, congenic, and intra-H-2-recombinant strains. Clin. Immunol. Immunopathol. 65, 98–109.

Hultman, P., Ganowiak, K., Turely, S.J., Pollard, K.M., 1995. Genetic susceptibility to silver-induced antifibrillarin autoantibodies in mice. J. Autoimmun. 77, 191–197.

Hultman, P., Turley, S.J., Eneström, S., Lindh, U., Pollard, K.M., 1996. Murine genotype influences the specificity, magnitude and persistence of murine mercury-induced autoimmunity. J. Autoimmun. 9, 139–149.

Izui, S., Kobayakawa, T., Zryd, M.J., Louis, J., Lambert, P.H., 1977. Mechanism for induction of anti-DNA antibodies by bacterial lipopolysaccharides in mice: II. Correlation between anti-DNA induction and polyclonal antibody formation by various polyclonal B lymphocyte activators. J. Immunol. 119, 2157–2168.

Jacobson, D.L., Gange, S.J., Rose, N.R., Graham, N.M.H., 1997. Epidemi-

ology and estimated population burden of selected autoimmune disease in the United States. Clin. Immunol. Immunopathol. 84, 223–243.

Johansson, U., Hansson-Georgiadis, H., Hultman, P., 1997. Murine silver-induced autoimmunity: silver shares induction of antinucleolar antibodies with mercury, but causes less activation of the immune system. Int. Arch. Allergy Immunol. 113, 432–443.

Johansson, U., Hansson-Georgiadis, H., Hultman, P., 1998. The genotype determines the B cell response in mercury-treated mice. Int. Arch. Allergy Immunol. 116, 295–305.

Kazantzis, G., Schiller, K.F.R., Asscher, A.W., Drew, R.G., 1962. Albuminuria and the nephrotic syndrome following exposure to mercury and its compounds. Q. J. Med. 31, 403–418.

Magos, L., 2001. Review on the toxicity of ethylmercury, including its presence as a preservative in biological and pharmaceutical products. J. Appl. Toxicol. 21, 1–5.

Magos, L., 2003. Neurotoxic character of thimerosal and the allometric extrapolation of adult clearance half-time to infants. J. Appl. Toxicol. 23, 263–269.

Magos, L., Brown, A.W., Sparrow, S., Bailey, E., Snowden, R.T., Skipp, W.R., 1985. The comparative toxicology of ethyl- and methylmercury. Arch. Toxicol. 57, 260–267.

Maibach, H., 1975. Acute laryngeal obstruction presumed secondary to thimerosal (Merthiolate) delayed hypersensitivity. Contact Dermatitis 1, 221.

Matheson, D., Clarkson, T., Gelfand, E., 1980. Mercury toxicity (acrodynia) induced by long-term injection of gamma globulin. J. Pediatr. 97, 153–155.

Mirtcheva, J., Pfeiffer, C., De Bruijn, J.A., Jaquesmart, F., Gleichmann, E., 1989. Immunological alterations inducible by mercury compounds: III. H-2A acts as an immune response and H-2E as an immune "suppression" locus for $HgCl_2$-induced antinucleolar antibodies. Eur. J. Immunol. 12, 2257–2261.

National Toxicology Program, 2003. Chemical Health and Safety Information: *O*-Carboxyphenyl-thio-ethylmercury Sodium Salt. National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Nielsen, J.B., 1992. Toxicokinetics of mercuric chloride and methylmercuric chloride in mice. J. Toxicol. Env. Health 37, 85–122.

Nielsen, J.B., Hultman, P., 2002. Mercury-induced autoimmunity in mice. Environ. Health Perspect. 110, S5877–S5881.

Pollard, K.M., Hultman, P., 1997. Effects of mercury on the immune system. In: Sigel, H., Sigel, A. (Eds.), Mercury and Its Effects on Environment and Biology. Marcel Dekker, Basel, pp. 421–440.

Pollard, K.M., Pearson, D.L., Hultman, P., Hildebrandt, B., Kono, D.H., 1999. Lupus-prone mice as models to study xenobiotic-induced acceleration of systemic autoimmunity. Environ. Health Perspect. 107, 729–735.

Pollard, K.M., Pearson, D.L., Hultman, P., Deane, T.N., Lindh, U., Kono, D.H., 2001. Xenobiotic acceleration of idiopathic systemic autoimmunity in lupus prone BXSB mice. Environ. Health Perspect. 109, 27–33.

Qvarnström, J., Lambertsson, L., Havarinasab, S., Hultman, P., Frech, W., 2003. 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. 75, 4120–4124.

Santucci, B., Cannistraci, C., Cristaudo, A., Camera, E., Picardo, M., 1998. Thimerosal positives: the role of organomercury alkyl compounds. Contact Dermatitis 38, 325–328.

Stratton, K., Gable, A., McCormick, M.C., 2001. Thimerosal-Containing Vaccines and Neurodevelopmental Disorders. Institute of Medicine, Washington, DC.

Suneja, T., Belsito, D., 2001. Thimerosal in the detection clinically relevant allergic contact reactions. J. Am. Acad. Pathol. 45, 23–27.

Suzuki, T., Miyama, T., Katsunuma, H., 1963. Comparative study of bodily distribution of mercury in mice after subcutaneous administration of methyl, ethyl, and *n*-propyl mercury acetates. Jpn. J. Exp. Med. 33, 277–282.

Suzuki, T., Takemoto, T.-I., Kashiwazaki, H., Miyama, T., 1973. Metabolic fate of ethylmercury salts in man and animal. In: Miller, M., Clarkson, T. (Eds.), Mercury, Mercurials and Mercaptans. Charles C. Thomas, Springfield, IL, pp. 209–232.

Takeuchi, K., Turley, S.J., Tan, E.M., Pollard, K.M., 1995. Analysis of the autoantibody response to fibrillarin in human disease and murine models of autoimmunity. J. Immunol. 154, 691–699.

Ulfvarson, U., 1962. Distribution and excretion of some mercury compounds after long term exposure. Int. Arch. Gewerbepathol. Gewerbehyg. 19, 412–422.

van't Veen, A.J., van Joost, T., 1994. Sensitization to thimerosal (Merthiolate) is still present today. Contact Dermatitis 31, 293–298.

Warfvinge, K., Hansson, H., Hultman, P., 1995. Systemic autoimmunity due to mercury vapor in genetically susceptible mice: dose–response studies. Toxicol. Appl. Pharmacol. 132, 299–309.

Westphal, G.A., Schnuch, A., Schulz, T.G., Reich, K., Aberer, W., Brasch, J., Koch, P., Wessbecher, R., Szliska, C., Bauer, A., Hallier, E., 2000. Homozygous gene deletions of the glutathione S-transferase MI and TI are associated with thimerosal sensitization. Int. Arch. Occup. Environ. Health 73, 384–388.

Wöhrl, S., Hemmer, W., Focke, M., Götz, M., Jarisch, R., 2003. Patch testing in children, adults, and the elderly: influence of age and sex on sensitization patterns. Pediatr. Dermatol. 20, 119–123.

Zenarola, P., Gimma, A., Lomuto, M., 1995. Systemic contact dermatitis from thimerosal. Contact Dermatitis 32, 107–123.