



Immunosuppressive and autoimmune effects of thimerosal in mice

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Received 9 July 2004; accepted 31 August 2004

Abstract

The possible health effects of the organic mercury compound thimerosal (ethylmercurithiosalicylate), which is rapidly metabolized to ethylmercury (EtHg), have recently been much debated and the effect of this compound on the immune system is largely unknown. We therefore studied the effect of thimerosal by treating A.SW (H-2^s) mice, susceptible to induction of autoimmunity by heavy metals, with 10 mg thimerosal/L drinking water (internal dose ca 590 µg Hg/kg body weight/day) for up to 30 days. The lymph node expression of IL-2 and IL-15 mRNA was increased after 2 days, and of IL-4 and IFN-γ mRNA after 6 and 14 days. During the first 14 days treatment, the number of splenocytes, including T and B cells as well as Ig-secreting cells decreased. A strong immunostimulation superseded after 30 days treatment with increase in splenic weight, number of splenocytes including T and B cells and Ig-secreting cells, and Th2- as well as Th1-dependent serum immunoglobulins. Antinucleolar antibodies (ANoA) targeting the 34-kDa nucleolar protein fibrillarin, and systemic immune-complex deposits developed. The H-2^s strains SJL and B10.S also responded to thimerosal treatment with ANoA. The A.TL and B10.TL strain, sharing background genes with the A.SW and B10.S strain, respectively, but with a different H-2 haplotype (*t1*), did not develop ANoA, linking the susceptibility to H-2. Thimerosal-treated H-2^s mice homozygous for the *nu* mutation (SJL-*nu/nu*), or lacking the T-cell costimulatory molecule CD28 (B10.S-CD28^{-/-}), did not develop ANoA, which showed that the autoimmune response is T-cell dependent. Using H-2^s strains with targeted mutations, we found that IFN-γ and IL-6, but not IL-4, is important for induction of ANoA by thimerosal. The maximum added renal concentration of thimerosal (EtHg) and inorganic mercury occurred after 14 days treatment and was 81 µg Hg/g. EtHg made up 59% and inorganic mercury 41% of the renal mercury. In conclusion, the organic mercury compound thimerosal (EtHg) has initial immunosuppressive effects similar to those of MeHg. However, in contrast to MeHg, thimerosal treatment leads in genetically susceptible mice to a second phase with strong immunostimulation and autoimmunity, which is T-cell dependent, H-2 linked and may at least partly be due to the inorganic mercury derived from the metabolism of ethyl mercury.

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Keywords: Thimerosal; Ethylmercury; Mice; Immunosuppression; Autoimmunity

Introduction

Thimerosal has for a long time been used as a wound disinfectant and a preservative in medical preparations, not least human vaccines (Magos, 2001). However, more extensive childhood immunization schedules and increased concern regarding the potential effect of low level exposure

of organic mercurials on neurodevelopment, recently raised the question of thimerosal in vaccines as a public health concern (Stratton et al., 2001a). As a precautionary measure, the use of thimerosal in vaccines has now been largely abandoned in the US (Ball et al., 2001).

Knowledge on the toxicokinetics and toxicology of thimerosal is limited (Clarkson, 2002), and to a large extent based on comparisons with methyl mercury (MeHg), which due to its presence as a common environmental contaminant has been more intensely studied (Stratton et al., 2001b). Thimerosal consists of an organic radical, ethylmercury (EtHg), bound to the sulfur atom of the thiol group of

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salicylic acid. Thimerosal contains 49.6% mercury by weight, and following tissue adsorption, EtHg rapidly dissociates from the thio-salicylic acid moiety and binds to the thiol ligands in tissue proteins (Magos, 2003). Identified effects of EtHg on the immune system are sparse. Thimerosal is a frequent skin sensitizer according to the patch test performed in patients with suspected contact allergy (Goncalo et al., 1996). However, the clinical relevance of sensitization is low (Suneja and Belsito, 2001), and thimerosal rarely causes systemic hypersensitivity (Maibach, 1975; Zenarola et al., 1995).

Based on the similarities between EtHg and MeHg with regard to chemistry, initial distribution in organisms and tissue (brain) damage (Clarkson, 2002), it is a plausible hypothesis that the effect of EtHg on the immune system is similar to that of MeHg. MeHg is a well-known immunotoxic substance (reviewed in (Descotes, 1986)). In vitro MeHg reduces T- and B-cell responses (Brown et al., 1988; Nakatsuru et al., 1985; Shenker et al., 1992, 1993). 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 responses in rodents (Brown et al., 1988; Hirokawa and Hayashi, 1980; Ohi et al., 1976) and may even cause atrophy of the immune system (Klein et al., 1972; Hirokawa and Hayashi, 1980).

More modest doses of MeHg (130–600 $\mu\text{g Hg/kg bw/day}$), comparable to those given in the present study, 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 (Ilbäck et al., 2000; Koller, 1975).

We recently reported (Havarinasab et al., 2004) that sufficient doses of thimerosal induce an autoimmune condition in genetically susceptible mice. This condition shared many characteristics with the autoimmune syndrome induced in such mice after exposure to inorganic mercury in the form of metallic mercury vapor (Warfvinge et al., 1995) or mercuric chloride via the oral (Hultman and Eneström, 1992) and the subcutaneous (Robinson et al., 1986) route. The autoimmune syndrome is characterized by lymphoproliferation with polyclonal B-cell activation and hypergammaglobulinemia (Pietsch et al., 1989; Pollard and Hultman, 1997), production of autoantibodies targeting the 34-kDa nucleolar protein fibrillarin (Hultman and Eneström, 1989; Reuter et al., 1989), and development of immune-complex deposits (Hultman and Eneström, 1988; Robinson et al., 1997). In the mouse, susceptibility to induction of antinucleolar/antifibrillarin antibodies (ANoA/AFA) with inorganic mercury is linked to the mouse MHC (H-2) haplotypes *s* and *q*, while most other haplotype are resistant to induction of ANoA/AFA (Hultman et al., 1992).

In this study, we assessed if thimerosal has immunosuppressive properties, and examined the relationship between

the immunosuppressive and autoimmune effects. Secondly, we studied cellular and molecular requirements for the autoimmune effect of thimerosal, including cytokine expression. Finally, we tried to link the effects on the immune system to the toxicokinetics of thimerosal.

Material and method

Mice. A.SW, B10.S (H-2^s) mice were obtained from Taconic M & B (Ry, Denmark). SJL/N mice (H-2^s) heterozygous (*nu/+*) or homozygous (*nu/nu*) for the nude mutation (Hultman et al., 1995a) were obtained from National Institute of Health (Bethesda, MD, USA) and bred in the animal facilities of the Faculty of Health Sciences, Linköping. Breeding pairs of A.TL and B10.TL mice (H-2^d) mice were obtained from Harlan Ltd. (Oxon, UK) and Department of Immunogenetics, University of Tübingen Germany, respectively, and breeding was maintained by sister–brother mating in the animal facilities of the Faculty of Health Sciences, Linköping. Breeding pairs of mice homozygous (*–/–*) for a targeted mutation (knock out—KO) of the gene for interleukin-4 (IL-4), interleukin-6 (IL-6), or CD28 on the background of B10.S mice (Kono et al., 1998; Pollard et al., 2003) were obtained from the Scripps Research Institute, La Jolla, CA, USA, and maintained by brother–sister mating in the animal facilities of the Faculty of Health Sciences, Linköping. B10.S-IFN- γ R^{–/–} mice (Pollard et al., 2003) were bred and kept at the Animal Department of the Scripps Research Institute, La Jolla, CA. All mice kept in Linköping were 8–12 weeks old at onset of the experiments, 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 SJL- *nu/+* and *nu/nu* mice were housed in isolators and given sterilized pellets and water. The pellets contained 23 ng Hg²⁺/g and 4 ng methyl mercury as Hg/g, whereas the EtHg concentration was below the detection limit (<0.1 ng/g) (Qvarnstrom et al., 2003).

Treatment. Thimerosal, C₉H₉HgNaO₂S (Fluka, Seelze, Germany), was given to groups of 4–8 mice as 10 mg/L drinking water. New solutions were prepared weekly by dissolving thimerosal in tap water. Controls received tap water without any additions. Groups of 5 A.SW mice were treated with thimerosal for 2.5, 6, 8.5, 14, or 30 days, and then killed by cervical dislocation. Five age- and gender-matched A.SW controls were also killed at each point of time. A.TL, B10.S, B10.TL, B10.S-IL-4^{–/–}, B10.S-IL-6^{–/–}, and B10.S-CD28^{–/–} mice were treated for 10 weeks, and SJL- *nu/+* and *nu/nu* mice for 4–10 weeks, with 10 mg thimerosal/L drinking water (Table 1). B10.S-IFN- γ R^{–/–} mice were treated with sc injection of 1.0 mg thimerosal/kg (internal dose ca. 333 $\mu\text{g Hg/kg b.w.}$) every third day for 4 weeks.

Blood and tissue sampling. A.SW mice were sacrificed after 2.5, 6, 8.5, 14, and 30 days and the following tissues were sampled: blood from the retroorbital plexus, mesenteric lymph nodes (mRNA extraction), the left kidney (speciation of mercury), and spleen (flow cytometric examination of lymphocytic phenotypes, activation markers, and cytoplasmic Ig-containing cells). In the 30-day treatment group, part of the mesenteric lymph nodes were in addition used for mercury speciation, and pieces of the spleen and the right kidney for examination of immune deposits. Blood was obtained from the retroorbital plexus in A.TL, B10.S, and B10.TL mice, the four strains of B10.S KO mice, as well as SJL-*nu*⁺ and *-nu/nu* mice after 4–6 weeks treatment. B10.S-IFN- γ R^{-/-} mice were sacrificed after 4 weeks, all other strains after 10 weeks treatment.

Ribonuclease protection assay (RPA). The mesenteric lymph nodes were carefully dissected, removed from the body and homogenized with an electric homogenizer (Omniion 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 manufacturer's instruction (Biotech Bulletin no. 28, 1993).

The Ribonuclease protection assay (RPA) has been described previously (Häggqvist 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 -³²P]UTP were transcribed from the template set mCK-1 (Pharmingen) with an in vitro transcription kit (Pharmingen). mRNA, 18–20 μ g, 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 h 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.).

Flow cytometry. Monoclonal antibodies (MAb) conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) targeting pan-T-cells CD3-FITC (clone A95-1), T-helper cells CD4-PE (GK1.5), pan-B-cells CD19-FITC (1D3), the very early activation marker CD69-PE (H1.2F3), transferrin receptor CD71-PE (C2F2), IL-2-

receptor CD122-PE (TM- β 1) and CD25-PE (3C7), as well as the Ig-isotypes IgE-FITC (R35-72), IgG1-FITC (A85-1), IgG2a-FITC (R19-15) and their isotype controls were obtained from Becton Dickinson (BD) (San Diego, CA, USA). The MAb to CD134-RPE (OX86) was obtained from Oxford Biotechnology Ltd. (Oxon, UK). RPMI-1640, Fetal Calf Serum (FCS), and Hanks' balanced salt solution (HBSS) 10 \times were obtained from Gibco (Paisley, UK). Rabbit serum was obtained from Dako (Copenhagen, Denmark). Permeabilizing agents (Perm/Wash and Cytofix/Cytoperm) for studies of intracellular antigens were obtained as kits from BD.

Spleen cell preparation. Single-cell suspensions were prepared and red blood cells lysed as described (Johansson et al., 1997a).

Two color flow cytometry analysis of cell surface markers. The splenic cell concentration was adjusted to 20 \times 10⁶ mononuclear cells/mL and 50 μ L (1 \times 10⁶ cells) was incubated with 40% rabbit serum for 20 min 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., 1997b). The data from 10,000 cells was then acquired and saved in list mode using an LSR flow cytometer (BD). Cells from control and thimerosal-treated mice were prepared and the data was 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.

Two color flow cytometry analysis of cytoplasmic Ig⁺ cells. To analyze cytoplasmic Ig⁺ cells (cIg⁺) 1 \times 10⁶ cells were incubated with 250 μ L of Cytofix/Cytoperm (BD) for 20 min 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 min. Washing with HBSS-2% FCS buffer was repeated twice, and the pellet was resolved in 1 mL Perm/Wash. Twenty thousand cells were acquired on the flow cytometer and saved in list mode.

Serum IgG1 concentrations assessed by ELISA. Analysis of serum IgG1 concentrations was performed as previously described (Johansson et al., 1997a). Briefly, microtiter plates (Nunc, Copenhagen, Denmark) were coated with rat anti-mouse IgG1 MAb (LO-IMEX, Brussels, Belgium).

The plates were washed and blocked. The wells were then incubated with diluted serum. Bound IgG1 was detected using horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG1 MAbs (LO-IMEX). The optical density in the wells was measured at 450 nm, and the background values obtained using PBS instead of serum were subtracted. Standard curves using mouse myeloma proteins of the IgG1 isotype (LO-IMEX) were used to obtain the actual concentration.

Serum IgG2a concentrations assessed by ELISA. Analysis of serum IgG2a concentrations was performed as previously described (Johansson et al., 1997a). 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).

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).

Serum anti-ssDNA antibodies assessed by ELISA. The method used has been described before (Johansson et al., 1997a). Microtiter plates (Nunc) were coated with single stranded DNA (ssDNA), followed by blocking and washing. Diluted sera were incubated in the wells. After repeated washing, wells were incubated with ALP-conjugated rabbit anti-mouse Ig (reacting with IgG, IgM and IgA) (Sigma). The optical density was measured at 405 nm, and the background values in wells coated with PBS were subtracted. A pool of sera from MRL-*lpr/lpr* mice was used as positive control. Using a monoclonal antibody from clone HB2 (SeraLab, Clowley Down, England), reacting with dsDNA, we were unable to detect any contamination with dsDNA (data not shown).

Serum anti-DNP antibodies assessed by ELISA. The method used has been described before (Johansson et al., 1997a). Microtiter plates (Nunc) were coated over human serum albumin conjugated with 30–40 moles DNP per mole albumin (Sigma). Following repeated washes, the wells were incubated with diluted sera, washed, and ALP-conjugated rabbit anti-mouse Ig (reacting with IgG, IgM,

and IgA) (Sigma) added. The optical density was measured at 405 nm, and the background values in wells coated with PBS were subtracted.

Serum antinuclear antibodies (ANA). For detection of serum ANA indirect immunofluorescence was performed as previously described (Hultman and Eneström, 1988) using sera diluted 1:40–1:80,960 which were incubated on slides with a monolayer of HEP-2 cells (Binding Site Ltd., Birmingham, UK), followed by goat anti-mouse IgG antibodies (Sigma Company, St. Louis, MO) (Fc-specific) diluted 1:50. A positive ANA test was defined as the highest serum dilution, which showed a detectable specific staining. No staining at a serum dilution of 1:40 was considered as a negative result. The pattern and titer of any antinuclear antibodies was assessed in each serum using a Nikon incident-light fluorescence microscope (Nikon Instech Co. Ltd., Kanagawa, Japan). All observations were done with coded samples. During the tests, pooled sera from young mice, which were individually found not to stain the cell nucleus or cytoplasm, were used as a negative control. With regard to pattern recognition, mouse sera characterized as containing antifibrillar antibodies by immunoblotting were used for antinucleolar pattern. For other ANA pattern human reference, sera were obtained from Binding Site, and FITC-conjugated anti-human IgG antibodies (Binding Site) were used as the second step reagent.

Tissue immune deposits. Pieces of the right kidney were examined with direct immunofluorescence as described before (Hultman et al., 1995b) 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 1:5,120. The highest dilution, which showed a specific fluorescence, was defined as the titer of the immune reactant. Pieces of the spleen were examined using anti-IgG and anti-C3c antibodies (abs) diluted 1:40. The presence of IgG and C3c deposits in the renal and splenic vessel walls was recorded and graded.

Speciation of mesenteric lymph node and renal mercury concentration. This method has previously been described by 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 μ L 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 DDTC into toluene and reacted with butylmagnesium chloride to form butylated derivatives. The derivatized species were sepa-

rated and detected by gas chromatography-inductively Coupled Plasma-mass spectrometry.

Statistics. Statistical analyses were performed using Graph-Pad Software Inc. Statistical differences between thimerosal-treated mice and controls were calculated using the non-parametric Mann–Whitney *U* test. The differences for ribonuclease protection assay (RPA) assessment were calculated using the non-parametric Kruskal–Wallis test followed by Dunn’s post-test. $P < 0.05$ was considered to be statistically significant.

Result

Mesenteric lymph node cytokine mRNA expression

A.SW mice treated with thimerosal for 2.5 days showed a significant increase in IL-2 and IL-15 mRNA expression compared with the controls (Fig. 1). After 6 days treatment, the expression of these two cytokines had declined and was not any longer significantly different from the controls. However, at this time the IFN- γ and IL-4 mRNA expression showed a 2- and 7-fold increase, respectively, which was significantly different from the controls (Fig. 1). The expression of IFN- γ and IL-4 mRNA then declined, but a

significant increase was seen again after 14 days treatment for both cytokines and for IL-4 after 30 days. The mRNA level of other cytokines (IL-9, IL-10, IL-13, IL-5, and IL-6) was close to the detection limits in both thimerosal-treated and control mice.

Effect of targeted mutations of cytokine and cytokine receptor genes

The effect of the above cytokines on thimerosal-induced autoimmunity was assessed by using genetically mercury-susceptible H-2^s mice (on the permissive C57BL/10 background) with targeted mutations of cytokines or cytokine receptor (Kono et al., 1998; Pollard et al., 2003). Sixty-seven percent of the mice homozygous for mutation of the IL-4 gene (B10.S-IL-4^{-/-}) showed a modest titer of ANoA after 6 weeks treatment with thimerosal, which was similar to the development of ANoA in identically treated B10.S mice with intact IL-4 gene (B10.S wild type mice) (Table 1). In comparison, only two out of eight thimerosal-treated B10.S mice with a homozygous mutation for the IFN- γ receptor gene (B10.S-IFN- γ R^{-/-}) showed traces of ANoA. B10.S mice with a homozygous mutation of the IL-6 gene (B10.S-IL-6^{-/-}) did not develop ANoA during treatment with thimerosal (Table 1).

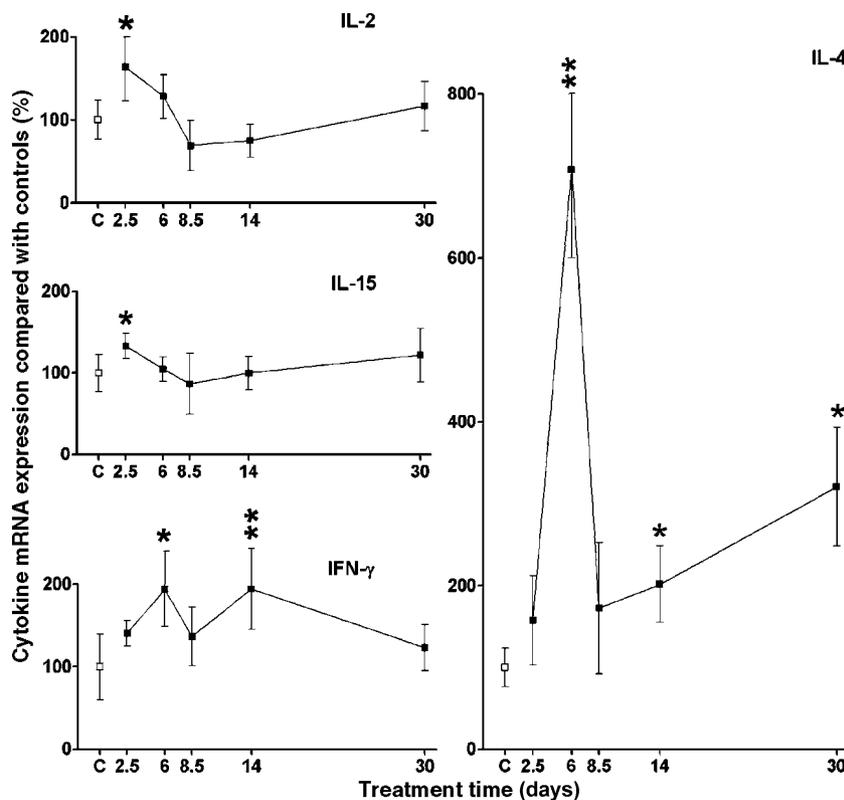


Fig. 1. Expression of cytokine mRNA in mesenteric lymph nodes in groups of five A.SW mice treated with 10 mg thimerosal/L drinking water for up to 30 days. The expression is shown as percentage of the mean cytokine expression in five control mice (C). The bars denote mean \pm SD. * $P < 0.05$, ** $P < 0.01$ significantly different from the controls using the Kruskal–Wallis test followed by Dunn’s post-test.

Table 1
Serum antinucleolar antibodies in mice treated with thimerosal

Strain	H-2	4–6 weeks		10 weeks	
		Positive/total ^a	Titer ^b	Positive/total ^a	Titer ^b
A.SW	<i>s</i>	8/8 ^c	18,400 ± 15,601	8/8	26,880 ± 26,639
A.TL	<i>t1</i>	0/4 ^c	0	0/4	0
SJL <i>nu</i> / ⁺	<i>s</i>	5/7 ^d	2432 ± 1717	4/8	1680 ± 1089
SJL <i>nu</i> / <i>nu</i>	<i>s</i>	0/6 ^d	0	0/5	0
B10.S	<i>s</i>	5/6 ^c	784 ± 507	4/6	640 ± 453
B10.TL	<i>t1</i>	0/7 ^c	0	0/7	0
B10.S IL-4 ^{-/-}	<i>s</i>	4/6 ^c	300 ± 248	3/6	320 ± 277
B10.S IL-6 ^{-/-}	<i>s</i>	0/4 ^c	0	0/4	0
B10.S CD-28 ^{-/-}	<i>s</i>	0/5 ^c	0	0/5	0
B10.S-IFN- γ R ^{-/-}	<i>s</i>	2/8 ^d	Traces	ND	ND

ND, not determined.

^a Fraction of positive mice.

^b Reciprocal mean ANoA titer ± SD in positive mice.

^c 6 weeks.

^d 4 weeks.

Total number of splenic mononuclear cells and splenic weight

Compared with the controls, the splenic weight of the A.SW mice was reduced by 13% after 2.5 days, but increased 21% after 6 days and 62–71% after 8.5–30 days (Table 2).

The total number of splenic mononuclear cells was reduced by 65% and 21% after 2.5 and 6 days thimerosal treatment, respectively, compared with the controls (Table 2). The number of these cells was marginally lower after 8.5 and 14 days in thimerosal-treated mice compared with the controls. After 30 days treatment, the number of splenic mononuclear cells was significantly higher (76%) than in the controls.

Number of splenic T-cells and expression of T-cell activation/proliferation markers

A.SW mice treated with thimerosal for 2.5, 6 and 14 days showed a significant 41–55% decrease in the number of pan T- (CD3⁺) cells and T-helper (CD4⁺) cells (Table 2). However, after 30 days treatment, the number of CD3⁺ and CD4⁺ cells was increased 54% and 67%, respectively, compared with controls. The expression of the very early activation markers CD69 and CD134 on CD3⁺ cells was not significantly different between thimerosal-treated mice and controls at any time (data not shown). The mean expression of the proliferation marker CD71 (transferrin receptor) on CD3⁺ cells was increased 22% and 28% compared with the controls after 2.5 and 14 days treatment with thimerosal, respectively, but the difference was not significant (data not shown). The mean expression of the IL-2-receptor marker CD122 decreased 8–9% after 6, 8.5, and 30 days thimerosal treatment, but increased 13% after 14 days compared with the controls (data not shown). The mean expression of the IL-2-receptor markers CD25 and CD 122 did not differ significantly at any time between thimerosal-treated mice and controls (data not shown).

Effect of mutations of T-cells and T-cell costimulatory molecules

The effect of deficient T-cell function on thimerosal-induced autoimmunity was assessed using mercury-susceptible H-2^s mice (SJL) homozygous for the nude mutation (*nu/nu*). These mice are deficient both with regard to the number of T-cells and the function of the few remaining T-cells (Dixon and Misfeldt, 1994; Hultman et al., 1995b; Kindred, 1978). None of 6 SJL-*nu/nu* mice developed ANoA during treatment with thimerosal for up to 10 weeks (Table 1). In contrast, SJL mice heterozygous for the *nu* mutation (SJL-*nu*/⁺), which have essentially normal T-cell phenotypes and a normal T-cell function (Hultman et al., 1995b), developed ANoA of a high titer. The importance of an intact T-cell activation for induction of ANoA with thimerosal was further assessed by using mercury-susceptible H-2^s mice with a targeted mutation for CD28 (B10.S-CD28^{-/-}). The T-cell costimulatory CD28 molecule, which induces T-cell proliferation and cytokine production after interaction with the B7 molecules (CD80 and CD86) if the T-cell receptor has reacted with an antigen presented in the context of MHC (Salomon and Bluestone, 2001), proved to be crucial for induction of ANoA with thimerosal (Table 1).

Number of splenic B-cells and expression of B-cell activation markers

A.SW mice showed a 51% and 33% decrease in the number of B- (CD19⁺) cells after 2.5 and 6 days treatment with thimerosal, respectively, compared with controls. After 8.5 and 14 days treatment the number of CD19⁺ cells was only marginally different from the controls, but a 67% increase was observed after 30 days (Table 1). The expression of the proliferation marker CD71 on B-cells was increased 4–38% during the first 14 days of treatment, but the difference was not significant compared with

Table 2
Total number of splenic lymphocytes, T- and B-cells and splenic weight in controls and thimerosal-treated A.SW mice

Treatment time (days)	Splenic weight		Total lymphocytes		CD3 ^a		CD4 ^a		CD19 ^a	
	Control	Thimerosal	Control	Thimerosal	Control	Thimerosal	Control	Thimerosal	Control	Thimerosal
	(mg)		($\times 10^6$)		($\times 10^6$)		($\times 10^6$)		($\times 10^6$)	
2.5	82.6 \pm 10.1	71.8 \pm 13.7	30.1 \pm 12.6	13.2 \pm 6.7*	8.9 \pm 3.2	4.0 \pm 1.7*	6.1 \pm 2.0	2.7 \pm 1.2*	10.2 \pm 3.7	4.2 \pm 2.7*
6	76.0 \pm 5.5	92.0 \pm 4.5**	37.0 \pm 6.4	29.3 \pm 3.9	14.0 \pm 1.9	9.0 \pm 1.5**	9.5 \pm 1.3	6.3 \pm 1.1*	14.8 \pm 3.2	9.8 \pm 0.7
8.5	71.6 \pm 3.6	116.2 \pm 8.3**	40.0 \pm 3.4	38.8 \pm 12.4	12.5 \pm 1.6	8.3 \pm 2.9	8.8 \pm 1.2	5.9 \pm 2.0	15.6 \pm 1.5	17.1 \pm 5.4
14	66.2 \pm 6.2	113.4 \pm 14.7**	32.3 \pm 8.2	30.6 \pm 8.1	12.2 \pm 2.1	7.2 \pm 1.9*	8.6 \pm 1.7	4.6 \pm 1.3*	12.1 \pm 3.1	11.8 \pm 5.9
30	124.8 \pm 44.3	205.6 \pm 57.6	47.1 \pm 12.7	82.8 \pm 15.4*	11.8 \pm 3.0	18.1 \pm 2.9*	7.2 \pm 1.8	12.0 \pm 2.4**	17.2 \pm 3.3	28.6 \pm 5.0*

Five animals were treated with thimerosal and 5 animals were used as controls; b.w., body weight.

^a Numbers are mean \pm SD.

* $P < 0.05$.

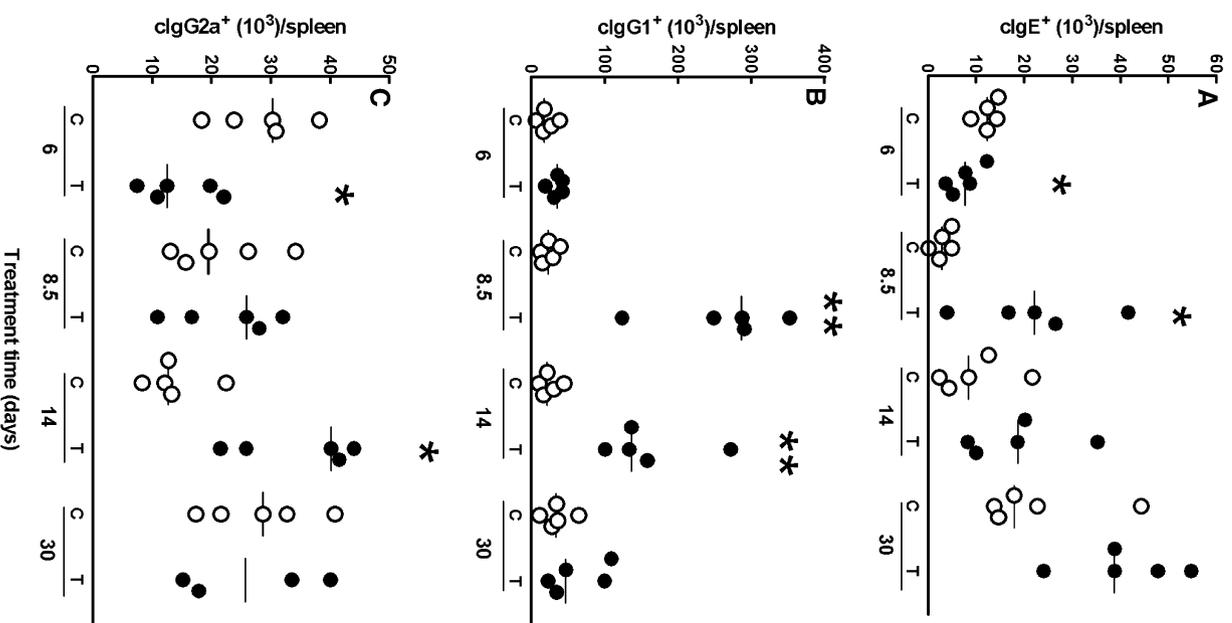


Fig. 2. The number of positive clg⁺ cells in the spleen of A.SW mice treated with 10 mg/L thimerosal (●) or untreated controls (○) for up to 30 days. C denotes control and T thimerosal-treated mice. Horizontal bars denote median number of positive clg⁺. * $P < 0.05$, ** $P < 0.05$ significantly different from controls (Mann–Whitney U test).

controls (data not shown). After 14 days, the expression of CD71 on CD19⁺ cells was similar compared with the controls, but a 16% decrease was observed after 30 days (data not shown).

Number of clg⁺ cells in the spleen

The mean number of clgE⁺ cells (Fig. 2A) was reduced by 40% after 6 days thimerosal treatment compared with controls, but increased 6-fold after 8.5 days treatment, and doubled after 14 and 30 days, compared with controls (Fig. 2A). After 8.5 and 14 days treatment with

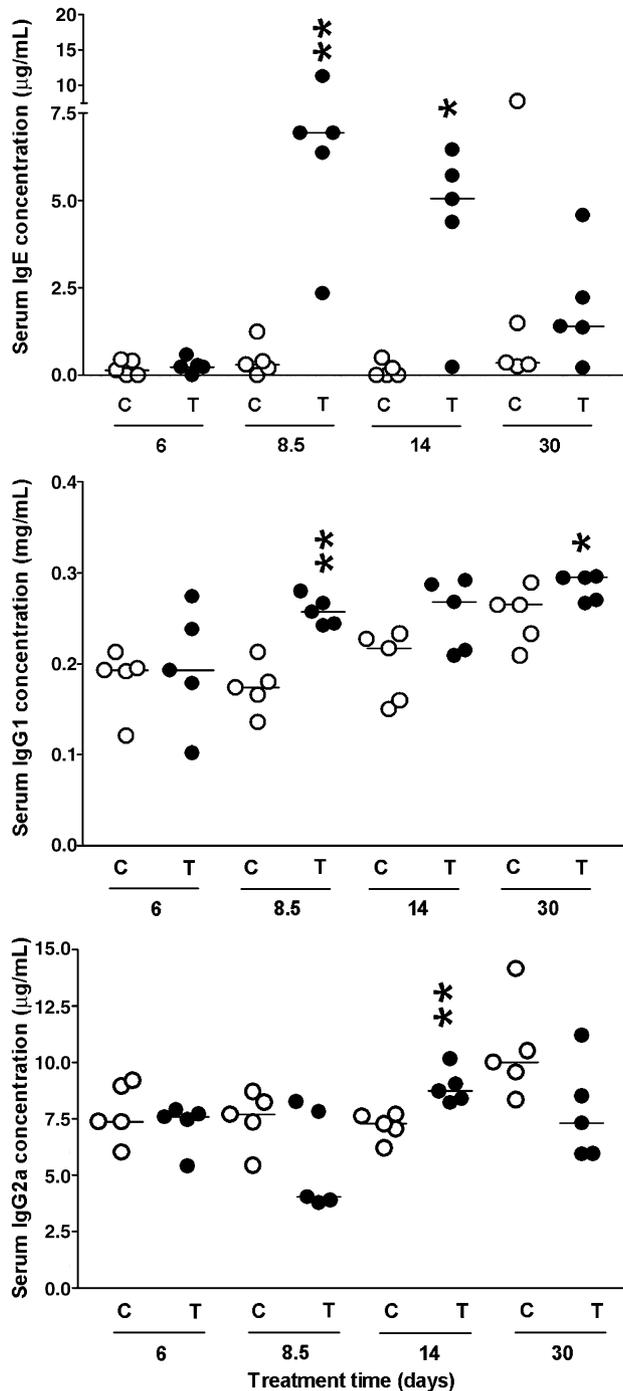


Fig. 3. Serum concentration of IgE, IgG1, and IgG2a in A.S.W mice treated with 10 thimerosal/L (●) or untreated controls (○). Horizontal bars denote median concentrations. * $P < 0.05$, ** $P < 0.01$ significantly different from controls (Mann–Whitney U test).

thimerosal, the number of cIgG1⁺ cells (Fig. 2B) was significantly increased 10- and 5-fold, respectively. The mean number of cIgG2a⁺ (Fig. 2C) cells was decreased 49% after 6 days thimerosal treatment but slightly increased after 8.5 days treatment compared with the controls. After 14 days treatment, the number of cIgG2a⁺ cells was increased 150%, but had returned to control values after 30 days.

Serum Ig concentrations

The mean serum IgE concentration was increased 15- and 30-fold after 8.5 and 14 days treatment with thimerosal, respectively, but returned to the control level after 30 days (Fig. 3). The mean serum IgG1 concentration was increased 8–48% after 8.5–30 days treatment. The mean serum IgG2a concentration was 7% and 26% lower than in the controls after 6 and 8.5 days thimerosal treatment, respectively, but 24% higher after 14 days treatment. After 30 days, thimerosal treatment the IgG2a concentration was 36% lower than in the controls (Fig. 3).

Polyclonal B-cell activation

Polyclonal B-cell activation, as measured by anti-DNP and anti-ssDNA antibodies, generally showed marginal differences between controls and thimerosal-treated mice. After 14 days thimerosal treatment, the anti-ssDNA and anti-DNP Abs titer were increased 60% and the difference was significant ($P < 0.05$) for the anti-ssDNA antibodies compared with controls (data not shown).

Serum antinuclear antibodies

After 6 and 8.5 days thimerosal treatment, the serum from a single A.S.W mouse in each group showed a faint staining of the nucleoli (Table 3). After 14 and 30 days treatment, sera from all mice caused a decoration of the nucleoli with bright granules (“clumpy staining”), a distinct staining of the condensed chromosomes in metaphase cells, and 2–6 brightly staining dots in the nucleoplasm. This staining pattern of antinucleolar antibodies (ANoA) corresponds to antifibrillar antibodies as evidenced by immunoblotting and immunoprecipitation (Hultman and Pollard, 1996). Another group of A.S.W mice showed after 6 weeks treatment with the same dose of thimerosal a higher mean ANoA titer, which increased further after 10 weeks treatment (Table 1). In contrast, A.TL mice (H-2^l) did not develop ANoA during treatment with thimerosal (Table 1). B10.S mice, with the H-2^s haplotype but C57BL/10 background genes, developed ANoA but with a lower titer than A.S.W mice. Mice on the C57BL/10 background but carrying the H-2 haplotype *tI* (B10.TL), did not develop ANoA during thimerosal treatment (Table 1).

Table 3
Serum antinucleolar antibodies in A.S.W mice treated with thimerosal

Treatment time (days)	Number	Positive/total	ANoA titer ^a
6	5	1/5	40 ± 0
8.5	5	1/5	40 ± 0
14	5	5/5	3358 ± 1717
30	5	5/5	11,264 ± 5609

^a Reciprocal mean ANoA titer ± SD in positive mice.

Tissue immune deposits

A.SW mice treated with 10 mg thimerosal/L drinking water for 30 days showed high titers of IgG and C3c deposits (data not shown). All mice showed renal vessel wall deposits of IgG, and 4/5 showed splenic vessel wall IgG deposits. However, none of the mice showed C3c deposits in the renal vessel walls, while 1/5 mice showed splenic vessel wall C3c deposits.

Tissue mercury concentration

Speciation of ethyl mercury and inorganic mercury (Hg^{2+}) showed that the EtHg concentration in the kidney had started to increase after 2.5 days thimerosal treatment, reached a maximum after 14 days (49 μg Hg/g tissue), and had marginally decreased after 30 days treatment. The concentration of Hg^{2+} was increased already after 2.5 days thimerosal treatment, and after 14 days, reached a peak level of 32 μg Hg/g tissue but declined by almost 50% after 30 days (Fig. 4). Finally, the speciation showed that the concentration of methyl mercury was always less than 2% of the total mercury concentration in the examined organs. Therefore, ethyl mercury is not significantly metabolized to methyl mercury in the organs, and methyl mercury does therefore not contribute to the immunosuppressive and immunostimulating effects observed after thimerosal treatment. The fraction of Hg^{2+} out of the sum of EtHg and inorganic mercury in the kidney was ca. 20% after 2.5 and 6 days, reached a maximum of 41% after 14 days, and declined to 29% after 30 days. Taken together, the maximum added renal concentration of EtHg and inorganic mercury was seen after 14 days when it reached 81 $\mu\text{g}/\text{g}$ tissue. The concentration of EtHg and Hg^{2+} in the mesenteric lymph nodes was after 30 days treatment 4.2 ± 0.4 and 1.8 ± 0.4 μg Hg/g (mean \pm SD), respectively. This corresponds to a fraction of Hg^{2+} of 30%. The controls showed concentrations of EtHg and Hg^{2+} below the

detection limits (<0.01 $\mu\text{g}/\text{g}$ tissue for EtHg and for Hg^{2+} <0.05 $\mu\text{g}/\text{g}$ kidney tissue and <0.15 $\mu\text{g}/\text{g}$ lymph node tissue).

Discussion

In a previous study (Havarinasab et al., 2004), we showed that a sufficient dose of thimerosal could induce all features of the mercury-induced autoimmune disease described after treatment with inorganic mercury in genetically susceptible mice (Pollard and Hultman, 1997). The present study concerns effects of thimerosal on cellular and humoral immunity, including cytokine expression, during development of autoimmune disease in such mice.

We found a reduced number of splenic T- and B-cells, including Ig-secreting cells, during the first 2 weeks of treatment with thimerosal, which indicates immunosuppression. We are not aware of any *in vivo* study except our own (Havarinasab et al., 2004) describing the effect of thimerosal on the intact immune system. Since thimerosal once taken up in the body is rapidly metabolized to EtHg, which has similar chemical properties and similar distribution as MeHg, the interaction of MeHg with the immune system is likely to be relevant also for the effect of thimerosal. High doses of MeHg (3,000–8,000 μg Hg/kg b.w.) cause an early reduction in the primary and secondary immune response and the proliferative capacity of T- and B-cells (Brown et al., 1988; Hirokawa and Hayashi, 1980; Ohi et al., 1976). However, doses of MeHg, similar to the dose of EtHg in the present study (590 μg Hg/kg bw/day), have seldom been studied. Blakley et al. (1980) reported, after 3 weeks treatment of Swiss mice with a dose of MeHg corresponding to 250 μg Hg/kg bw/day, a 57% and 25% reduction of the primary and secondary immune response, respectively. In addition, we recently found that MeHg treatment of A.SW mice using a daily dose of Hg very similar to the one in the present study caused an initial, transient reduction of lymphocytes, especially B-cells (Häggqvist et al., unpublished observations).

In contrast, sc injections of A.SW mice with inorganic mercury in the form of HgCl_2 corresponding to 50% of the dose of Hg given in the present study, did not cause any initial reduction but instead an increase of splenic T- and B-cells starting after 3 and 14 days treatment, respectively (Johansson et al., 1998). CD-1 mice treated for 28 days with a 3-fold higher dose of Hg in the form of inorganic Hg, compared with the dose of thimerosal in the present study, showed no consistent change in splenic T- and B-cells populations (Brunet et al., 1993). Finally, the murine B-cell response was unaffected after 7 weeks treatment with a 50% higher dose of Hg as HgCl_2 (Dieter et al., 1983).

These *in vivo* studies clearly show that MeHg is a much more potent immunosuppressive mercury compound than inorganic mercury, which concurs with a number of *in vitro*

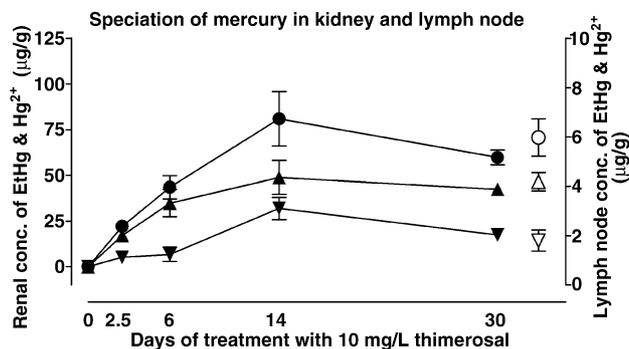


Fig. 4. Renal and lymph node mercury speciation in kidneys and lymph nodes from A.SW mice treated with 10 mg/L thimerosal using gas chromatography-inductively coupled plasma-mass spectrometry. (●)renal EtHg + Hg^{2+} , (▲) renal EtHg, (▼)renal Hg^{2+} , (○) EtHg + Hg^{2+} in lymph nodes, (△) EtHg in lymph nodes, (▽) Hg^{2+} in lymph nodes. The bars denote mean \pm SEM.

studies (Nakatsuru et al., 1985; Shenker et al., 1992, 1993). Our study clearly indicates that EtHg is similar to MeHg with respect to the immunosuppressive effect on the immune system *in vivo*.

We also show in this study that the immunosuppressive effect of thimerosal in A.SW mice is superseded by lymphoproliferation as evidenced by increased splenic weight and number of splenocytes. 30 days after onset of treatment the number of T- and B- cells had increased 54% and 67%, respectively. Similar to the autoimmune disease induced by inorganic mercury (Häggqvist and Hultman, 2001; Johansson et al., 1998), thimerosal induced a distinctly increased expression of IL-4 mRNA and a large increase in Th2-dependent Ig-secreting cells and serum immunoglobulins. The increase in IL-4 has been attributed to a direct induction of IL-4 gene expression in lymphocytes by Hg (Badou et al., 1997). We also found that the Th1-dependent IgG2a isotype was increased after 14 days thimerosal treatment which concurred with the increased IFN- γ mRNA expression. The increase of IFN- γ mRNA was more protracted and forceful than after treatment with inorganic mercury (Häggqvist and Hultman, 2001).

The increased expression of IL-2, IL-15, IL-4, and IFN- γ mRNA occurred during the first 6–14 days of thimerosal treatment, when the number of splenic T- and B-cells was reduced (present study). This might seem as a paradox. However, since the mRNA expression is normalized to the “house-keeping” gene GAPDH, our data demonstrate that the mean cytokine mRNA expression in the lymph node cells was increased. Our findings are therefore fully compatible with a reduction of the total number of (T-) lymphocytes, provided that at least a fraction of the remaining lymphocytes responded with an increased expression of cytokine mRNA. It might be hypothesized that the early increase of cytokine mRNA, especially IFN- γ , is a prerequisite for the subsequent strong immunostimulation and autoimmunity after thimerosal treatment (present study), since MeHg treatment leads to an increase of only IL-4 mRNA expression, which is followed by a weak autoimmune reaction with only marginal immunostimulation (Häggqvist et al., unpublished observations).

Thimerosal treatment for 14 days induced not only lymphoproliferation, but also antinucleolar antibodies (ANoA). The thimerosal-induced ANoA in A.SW mice have previously been shown to target the 34-kDa protein fibrillarlin (Havarinasab et al., 2004). We show in this study that A.TL mice with the same background genes as A.SW mice but bearing the H-2^l haplotype (K^s A _{β} ^k A _{α} ^k E _{β} ^k E _{α} ^k S^k D^k) did not develop ANoA, linking the susceptibility to develop ANoA after thimerosal treatment to the H-2 region to the right of the K locus. This is in agreement with findings after treatment with inorganic mercury (Hultman et al., 1992). Since H-2^s mice homozygous for the *nu* mutation (nude mice), or with a targeted mutation of the gene for CD28 (a co-stimulatory molecule for T-cells), did not

develop ANoA, the autoimmune reaction induced by thimerosal is clearly dependent on T-cells as shown for inorganic mercury (Hultman et al., 1995b; Pollard et al., 2003). Furthermore, the strength of the ANoA response after thimerosal treatment was determined by background (non-H-2) genes, and the order of magnitude was A.SW>SJL>B10.S, which is also in agreement with observations after treatment with inorganic mercury (Nielsen and Hultman, 2002).

The importance of the cytokines for which we assessed mRNA, with regard to the autoimmune disease, was examined by using genetically metal-susceptible mice with targeted mutations. Induction of autoimmunity by thimerosal was not affected by a lack of IL-4, which indicates that it is not a Th2-dependent disease as already demonstrated for the autoimmunity induced by inorganic mercury (Kono et al., 1998). Thimerosal treatment of IFN- γ R^{-/-} mice for 4 weeks caused only traces of ANoA in a few animals. This indicates that the IFN- γ /IFN- γ R pathway is vital for ANoA development, which would be in agreement with findings for inorganic mercury (Kono et al., 1998). Expanding the treatment time from 4 to 10 weeks in SJL-*nu*/+ mice increased neither the fraction of mice with ANoA nor the titer. In addition, increasing the treatment time from 6 to 10 weeks in A.SW and B10.S mice did not significantly increase ANoA. These observations make it highly unlikely that prolonged the treatment of IFN- γ R^{-/-} mice beyond 4 weeks would have enhanced ANoA development. However, this cannot be formally excluded.

H-2 mice lacking IL-6 did not show any ANoA response to thimerosal, which is at variance with the preserved ANoA response after treatment with inorganic mercury in these mice (Pollard et al., 2003). The reason for the different response of IL-6^{-/-} mice to these two mercury species is unknown. However, the IL-6-deficient mice treated with inorganic mercury differed from the identically treated wild-type mice showing reduced IgG and IgM hypergammaglobulinemia, and loss of the antichromatin autoantibody response (Pollard et al., 2003, 2004). The suppression of the Ig response is expected since IL-6 is important in B-cell differentiation and plays a crucial role in T-cell-dependent and -independent polyclonal IgG production (Markine-Goriaynoff et al., 2001). A selective loss of the antichromatin (anti-DNA) autoantibody response in IL-6 deficient mice has also been observed in the pristine-induced model of systemic autoimmunity (Richards et al., 1998). While the mechanisms behind the selective loss of these autoantibody responses are unclear, it should be noted that IFN- γ influences the expression and function of IL-6 (Boehm et al., 1997). Therefore, the attenuated autoantibody response observed in IL-6-deficient mice may be related to attenuation of down-stream events following the IFN- γ /IFN- γ receptor interaction that promotes development of HgIA (Kono et al., 1998).

Thimerosal treatment has been described to result in a qualitatively similar disposition of EtHg in the tissues as

compared with MeHg (Clarkson, 2002). However, from a quantitative point of view studies in mice have shown higher levels of EtHg in the kidney and liver, but lower levels in the brain (Suzuki et al., 1963). A long-term study in rats showed higher concentration of EtHg in the kidney but reduced levels in the brain as compared with MeHg (Ulfvarson, 1962). We found a maximum EtHg concentration of 49 $\mu\text{g MeHg/g}$ in the kidney after 14 days treatment, which was followed by a marginal decrease on day 30. This is close to the maximal concentration of 45 $\mu\text{g MeHg/g}$ after 30 days treatment with similar daily doses of Hg as MeHg (Häggqvist et al., unpublished observations). The continuous increase of the MeHg concentration for up to at least 30 days is in accordance with a $t_{1/2}$ for MeHg in mice of 7–12 days (Nielsen and Andersen, 1991), and a need for five $t_{1/2}$ to reach steady-state (WHO, 1990). Therefore, the steady-state level for MeHg should be reached after a minimum of 35 days. The maximum concentration of EtHg after 14 days in the present study indicates that the $t_{1/2}$ for EtHg is shorter than for MeHg. This may partly be explained by a more rapid conversion of EtHg to inorganic mercury compared with MeHg (Magos et al., 1985; Matheson et al., 1980).

With regard to the fraction of total mercury present as inorganic mercury after thimerosal treatment, Suzuki et al. (1973) reported 17–21% Hg^{2+} in the kidneys 6 days after a single injection of thimerosal. A similar fraction of Hg^{2+} was seen in the kidneys of our mice after 6 days continuous peroral treatment. We found a maximum fraction of inorganic mercury (41%) after 14 days thimerosal treatment. A comparable dose of Hg given as MeHg for up to 30 days caused a continuous increase in the fraction of inorganic mercury, which however reached only 22% after 30 days treatment (Häggqvist et al., unpublished observations). Finally, when equipotent doses of Hg were given as thimerosal or MeHg for up to 30 days, the maximum total kidney Hg concentration was higher after thimerosal as compared with MeHg treatment. The presence of a substantial fraction of inorganic mercury in the tissues of thimerosal-treated mice, and the many similarities between the immunostimulation which develops after thimerosal treatment and primary treatment with inorganic mercury (Pollard and Hultman, 1997), indicates that inorganic Hg may be responsible for the immunostimulatory effect. The threshold for induction of HgIA using inorganic Hg is around 4 $\mu\text{g/g}$ tissue (Hultman and Nielsen, 2001), a threshold which was rapidly reached in thimerosal-treated mice. However, this observation does not exclude the possibility that EtHg also contributed to the stimulatory effect.

In conclusion, treatment of genetically metal-susceptible mice with the organic mercury compound thimerosal (EtHg) has initially a similar suppressive effect on the immune system as MeHg. However, thimerosal treatment subsequently leads to strong immunostimulation and autoimmun-

ity, which is at variance with only a weak autoimmune response after MeHg treatment.

Acknowledgments

This study was supported by a grant from the Swedish Research Council, Branch of Medicine (project no. 09453). The technical assistance of Elham Nikookhesal and Marie-Louise Eskilsson is gratefully acknowledged.

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