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Mercury-induced renal immune complex deposits in young (NZB × NZW)F_1 mice: characterization of antibodies/autoantibodies

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SUMMARY

It is well demonstrated that mercury induces a systemic autoimmune disease in susceptible mouse strains. One of the major characteristics of mercury-induced autoimmune disease in mice is the development of renal immune complex deposits. We have previously shown that continual injection of mercury into young autoimmune prone (NZB × NZW)F_1 mice induced an increase in antibody/autoantibody production as well as development of early renal immune complex deposits. In the present study, we characterized the isotype, the specificity and the possible pathogenicity of deposited immunoglobulins in the kidneys of mercury-injected (NZB × NZW)F_1 hybrids. We found that young (NZB × NZW)F_1 mice injected with mercuric chloride ($HgCl_2$) for 6 weeks developed intense antibody formation of all immunoglobulin isotypes (except for IgG2b) as well as high levels of granular deposits of IgM, IgG1, IgG2a and IgG3 antibodies in the renal mesangium. Increased levels of the same antibody isotypes were also found in the kidney eluate of mercury- but not saline-injected mice. The dominant antibody in the kidney eluate of mercury-injected mice was of IgG1 isotype and found to be directed against double-stranded DNA, collagen, cardiolipin, phosphatidylethanolamine, and the hapten trinitrophenol, but not against nucleolar antigens. Further studies demonstrated that mercury-induced renal immune complex deposits in young (NZB × NZW)F_1 mice did not lead to a severe kidney injury. Thus, in response to mercury, young (NZB × NZW)F_1 mice develop renal immunoglobulin deposits with an isotype and specificity pattern correlating with that seen in the spleen and in the serum.

Keywords mercury (NZB × NZW)F_1 mice renal immune complex deposits antibodies autoantibodies

INTRODUCTION

Mercury is a heavy metal ion which, induces a systemic autoimmune disease in susceptible mice and rats (reviewed in [1–3]). In highly susceptible mice, the mercury-induced autoimmune disease is characterized by a T cell-dependent polyclonal B cell activation, by mainly increased serum levels of IgG1 and IgE antibodies, by the production of anti-nucleolar autoantibodies (ANolA) and by the formation of renal immune complex deposits [1–3].

The susceptibility to mercury-induced autoimmune disease varies among inbred mouse strains [1–5]. Highly susceptible mouse strains such as SJL (H-2^s), ASW (H-2^s), B10S (H-2^s) exhibit all mercury-induced autoimmune manifestations (see above), whereas the highly resistant mouse strain DBA/2 (H-2^d) does not develop any of these manifestations ([1–5] and our own unpublished observations). So far, other tested inbred mouse strains have been found to show one or more of the mercury-induced autoimmune characteristics ([4,5] and our own unpublished observations). These findings led to the suggestion that mercury-induced autoimmune disease in mice is genetically controlled and that MHC genes and other unknown non-MHC genes play a role [1–5]. However, further and more detailed studies are needed to characterize the genes that determine susceptibility/resistance to mercury-induced autoimmune disease.

We have previously studied effects of mercury in young autoimmune-prone (NZB × NZW)F_1 (H-$2^{d/z}$) mice [6]. We found that these mice, prior to development of spontaneous systemic autoimmune disease, were highly susceptible to mercury-induced autoimmune disease [6]. In fact, chronic injection with subtoxic doses of mercuric chloride ($HgCl_2$) into young (NZB × NZW)F_1 mice resulted in the development of all the above mentioned mercury-induced autoimmune manifestations except for anti-nucleolar antibody (ANolA) production [6].

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One of the striking characteristics of mercury-induced immunological alterations in young (NZB×NZW)F_1 mice was the early formation of high titres of immune complex deposits, mainly of IgG1 antibodies in the kidneys [6]. It has been shown that in highly susceptible SJL mice, which produce high levels of circulating ANolA and develop renal immune complex after injection with mercury, ANolA formed the major part of the renal immune complex deposits [7]. Since in young (NZB×NZW)F_1 mice, the development of mercury-induced renal immune complex deposits occurred in the absence of ANolA production [6], it was possible that autoantibodies with other specificities were involved in the formation of immune complex deposits in mercury-injected young (NZB×NZW)F_1 mice. The main aim of this study was to test this possibility. We performed experiments to analyse and to characterize the isotype and specificity of the deposited antibodies in the kidneys of mercury-injected young (NZB×NZW)F_1 mice. We also studied whether mercury-induced renal immune complex deposits had pathogenic significance.

MATERIALS AND METHODS

In vivo *treatment with $HgCl_2$*

A solution of 0·4 mg/ml $HgCl_2$ (analytical grade; Merck, Darmstadt, Germany) was prepared in sterile saline. A group of (NZB×NZW)F_1 mice (five to six mice per group) were injected subcutaneously with 0·1 ml of $HgCl_2$ solution (1·6 mg/kg body weight) twice a week for 6 weeks. The control group (five to six mice per group) only received 0·1 ml of sterile saline by s.c. route. Both mercury- and saline-injected mice were 5–8 weeks old at the beginning of the experiment. The experiment was repeated four times and similar treatment procedures were used in each experiment.

Urine, blood, kidney and spleen collection

At the end of each experiment, mercury- and saline- injected mice were bled by retroorbital puncture under light ether anaesthesia. Thereafter, the same mice were killed by cervical dislocation. Their urine samples were collected in 90 mm diameter, polystyrene Petri dishes (Labora, Upplands Väsby, Sweden) and their kidneys and spleens were removed. The blood from each mouse was allowed to clot at 4 °C and serum was separated after centrifugation. The sera and kidneys were stored at −20 °C before use. The spleens were directly tested for the presence of antibody-secreting cells.

Determination of urinary protein

Urine samples from mercury- and saline-treated mice were analysed for the presence of albumin using a standard dipstick (Ecur^4 Test; Boehringer, Mannheim, Germany). Mice were considered to have developed significant proteinuria when the reading for albumin was ≥1 (0·3 g/*l*).

Protein A plaque assay

Splenic antibody-secreting cells of different immunoglobulin classes and subclasses were enumerated by using a protein-A plaque assay as described by Gronowicz *et al.* [8]. Rabbit anti-mouse IgM, IgG1, IgG3 (Organon Teknika, Durham, NC) and IgG2b (Nordic Immunological Laboratories, Tilburg, The Netherlands) were used as developing reagents. Since proper developing reagents for enumeration of IgG2a and IgE antibody-secreting cells were not available, we used ELISA methods to measure these antibodies in serum (see below).

Detection of renal immune complex deposits

The presence of glomerular deposits of IgM, IgG1, IgG2a, IgG2b, IgG3 antibodies and/or complement factor C3 was detected by direct immunofluorescence, as described previously [6]. Briefly, the acetone-fixed kidney cryostat sections were incubated with serial dilutions of either FITC-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 antibodies (all from Southern Biotechnology, Birmingham, AL) or goat anti-mouse complement factor C3 (Cappel, Organon Teknika). The initial dilution for FITC-conjugated antibodies was 1:40. The highest dilution of the antibodies at which a specific fluorescence could be seen was defined as the titre of the glomerular deposits.

Eluting procedure

Kidneys from mercury- and saline-treated (NZB×NZW)F_1 mice were thawed and then elution was performed in acid buffer as described by Bowman *et al*. [9]. Briefly, the kidneys were minced with forceps, forced through a stainless steel grid and repeatedly washed with cold PBS. After examination by light microscopy, the isolated glomeruli were suspended in cold PBS and sonicated by a sonicator (Sonifier; Branson Sonic Power Co, Danbury, CT) in 30-s bursts. Sonication was continued until all glomeruli were disrupted (as examined by light microscopy). The sonicated glomeruli were washed in cold PBS at 700 ***g*** until the optical density (OD) at 280 nm of the supernatants was < 0·02. Thereafter, deposited antibodies were eluted by embedding the sonicated glomeruli in 3 ml of 0·1 M glycine–HCl pH 2·8 for 20 min with gentle stirring at 4°C. The eluates were centrifuged at 700 ***g*** for 5 min and then immediately neutralized with 1 M Tris buffer. The buffer of the neutralized eluates was changed to PBS using PD10 column (Pharmacia, Uppsala, Sweden). In a typical experiment, protein concentrations of the eluates from the kidneys of mercury- and saline-treated (NZB×NZW)F_1 mice were 70 μg/ml and 60 μg/ml, respectively.

ELISA for mouse IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies

Total mouse serum IgG2a antibodies and the presence of mouse IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies in the kidney eluates were determined by using ELISA techniques. Micro-ELISA plates (Costar, Cambridge, MA) were coated with 50 μl/well goat anti-mouse immunoglobulin (Southern Biotechnology) at 20 μg/ml in carbonate buffer pH 9·8 and were incubated overnight at 4°C. Plates were washed with tap water and unbound sites in each well were blocked by incubation with 50 μl 1% bovine serum albumin (BSA) in PBS at room temperature for 2 h. Plates were washed three times with PBS–Tween 20, then 50 μl of serially diluted kidney eluates (starting with 1:1) or mouse IgM (The Binding Site, Birmingham, UK), IgG1, IgG2a, IgG2b and IgG3 standards (all purchased from PharMingen, San Diego, CA) were added to the wells. Plates were incubated overnight at 4°C. Thereafter, plates were washed four times with PBS–Tween 20 and 50 μl 0·4 μg/ml alkaline phosphatase-labelled goat anti-mouse IgM, IgG1, IgG2a, IgG2b and/or IgG3 (Southern Biotechnology) were added to each well. After a 2-h incubation at 37°C, plates were washed three times with PBS–Tween 20 and 50 μl 1 μg/ml *p*-nitrophenyl phosphate in diethanolamine buffer were added to

each well. After 10–20-min incubation at room temperature, colour reaction was quantified by an ELISA reader (Anthos Labtec Instruments, Salzburg, Austria) at 405 nm.

ELISA for mouse IgE antibody

The presence of IgE antibodies in the sera, kidney eluates and in the kidney pellets was determined by a sandwich ELISA assay as described previously [6]. We used a rat anti-mouse IgE MoAb, R35-72 (PharMingen, San Diego, CA, USA) as a primary antibody and a biotinylated rat anti-mouse mAb, R35-79 (PharMingen) as secondary antibody.

Detection of ANolA

The presence of IgG1 and/or IgG2a ANolA in the kidney eluates was determined by indirect immunofluorescence using rat liver sections as a substrate [6]. The initial dilution for the kidney eluates was 1:1. Serum from a SJL mouse injected with mercury for 4 weeks was used as a positive control. The highest kidney eluate dilution at which nucleolar fluorescence could be detected was defined as the titre of IgG1 and/or IgG2a ANolA.

ELISA for detection of autoantibodies

The specificities of IgG1 and/or IgG2a antibodies present in the kidney eluates were determined by ELISA techniques as previously described [6]. Briefly, 50 μl/well of serially diluted kidney eluates (starting with 1:1) were added to micro-ELISA plates (Costar) coated with collagen type VII, cardiolipin (CL), phosphatidylethanolamine (PE), bovine thyroglobulin (all from Sigma Chemical Co., St Louis, MO), calf thymus double-stranded (ds)DNA (Serva, Heidelberg, Germany) and trinitrophenol conjugated to BSA (TNP–BSA). Following overnight incubation at 4°C, plates were washed three times with PBS–Tween and 50 ml alkaline phosphatase-labelled goat anti-mouse IgG1 and/or IgG2a (Southern Biotechnology) were added to each well as a detecting reagent. After a 2-h incubation at 37°C, plates were washed and 50 ml substrate were added to each well. After 60–90 min, absorbance was read at 405 nm.

Statistical analysis

Numbers of antibody-secreting cells of different isotypes, serum IgG2a and IgE levels and titres of glomerular deposits of IgM, IgG1, IgG2a, IgG2b, IgG3 and complement (C3) were shown as the means $\pm$ 1 s.d. The differences between these parameters in mercury- and in saline-injected mice were analysed with the Wilcoxon–Mann–Whitney test.

RESULTS

Induction of antibody production in young (NZB×NZW)F_1 mice by mercury

In our previous study [6], we found that in young (NZB×NZW)F_1 mice, mercury-induced antibody production peaked 6 weeks after treatment with mercury. Therefore, groups of young (NZB×NZW)F_1 mice were regularly treated with either $HgCl_2$ or saline for 6 weeks. At the end of the experiment, spleens and sera were tested for the presence of different immunoglobulin antibody-secreting cells and antibody levels, respectively. We also measured serum IgG2a levels, which were not included in the previous study. As shown in Fig. 1a,b, mice treated with mercury exhibited high numbers of splenic IgG1, IgG3 and IgM antibody-secreting cells as well as high levels of serum IgG2a and IgE antibodies when compared with saline-treated control mice. It should be emphasized that in response to mercury, young (NZB×NZW)F_1 mice exhibited a dominant and highly significant increase ($P < 0{\cdot}0001$) in IgG1 antibody production (Fig. 1a).

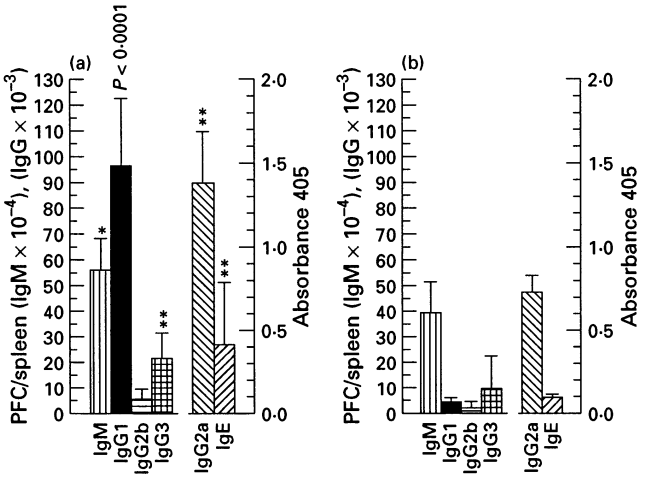

Fig. 1. Mercury induces antibody formation of different isotypes in young (NZB×NZW)F_1 hybrids. Groups of (NZB×NZW)F_1 mice (five to six mice/group) were repeatedly injected subcutaneously with either $HgCl_2$ (a) or saline (b) for 6 weeks. At the end of experiment, spleens were tested for IgM, IgG1, IgG2b and IgG3 antibody-secreting cells and the sera were tested for IgG2a and IgE antibodies by using protein A plaque assay and ELISA methods, respectively. Data are shown as mean + 1 s.d. Significant differences between mercury- and saline-injected mice were calculated by Wilcoxon–Mann–Whitney test. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

Analysis of renal immune deposits

In the next step, we performed an experiment to examine whether the mercury-induced circulating antibodies in young (NZB×NZW)F_1 mice were able to accumulate in the kidney. To do this, kidney sections from the same experimental mice (Fig. 1) were tested for the deposited IgG1, IgG2a, IgG2b, IgG3 and complement factor C3 (C3). As depicted in Fig. 2a,b, young (NZB×NZW)F_1 mice injected with mercury showed an increase in the titres of granular deposits of IgM, IgG2a, IgG3 and IgG1 antibodies in the renal mesangium compared with saline-injected controls. No significant differences were observed between mercury- and saline-injected mice with regard to titres of deposited IgG2b antibody and C3 in the glomeruli (Fig. 2a,b). This finding clearly demonstrated that the renal deposition of IgM, IgG1, IgG2a and IgG3 antibodies corresponds to the increased formation of these antibodies in the spleens and sera of mercury-injected (NZB×NZW)F_1 hybrids.

Analysis of kidney eluates

To confirm the above findings and to characterize further the deposited antibodies in the kidney of mercury-treated (NZB×NZW)F_1 mice, accumulated antibodies in the kidneys of the same experimental mice as described in Fig. 2 were eluted in acid conditions and their isotypes were determined. As shown in Fig. 3a,b, the acid eluate from the kidneys of mercury-injected mice contained much higher levels of IgM, IgG1, IgG2a and IgG3 antibodies compared with those of saline-injected mice. As expected, the dominant antibody in the kidney eluate of mercury-injected mice was of IgG1 isotype (Fig. 3a). However, kidney

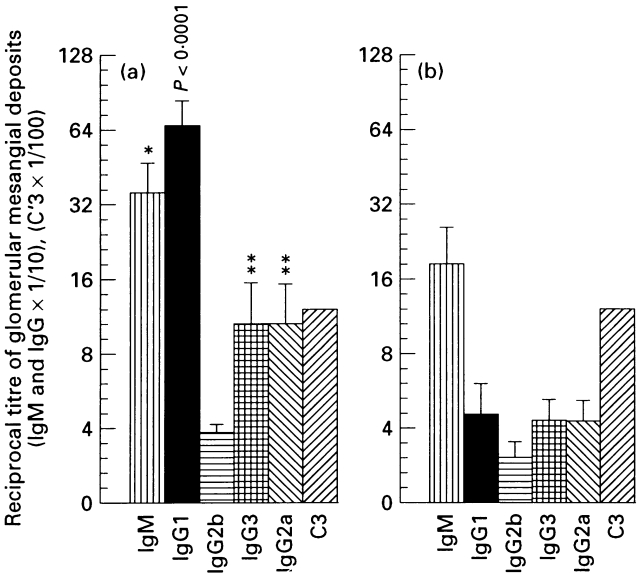

Fig. 2. Induction of renal deposits of various immunoglobulin isotypes in young (NZB×NZW)F$_1$ mice by mercury. Kidneys form the same young (NZB×NZW)F$_1$ mice (as described in Fig. 1) treated with either mercury (a) or saline (b) were analysed for the presence of glomerular deposits of IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies as well as complement factor C3 by direct immunofluorescence methods. Significant differences between the titres of granular mesangial IgM, IgG1, IgG2a, IgG2b, IgG3 and C3 in mercury- and saline-injected mice were calculated by Wilcoxon–Mann–Whitney test. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

eluate of mercury-injected mice did not show any significant increase in levels of IgG2b and IgE antibodies compared with those of saline-injected controls (Fig. 3a,b).

Evaluation of antibody specificities in kidney eluates

In our previous study, we demonstrated that the circulating IgG1 antibodies in young (NZB×NZW)F$_1$ mice reacted against different endogenous and exogenous antigens such as dsDNA, IgG, collagen, CL, PE and TNP [6]. Since the dominant antibody eluted from the kidneys of mercury-injected (NZB×NZW)F$_1$ mice was also of IgG1 isotype, in the next step we performed an experiment to test the specificities of IgG1 antibodies in the kidney eluates. High levels of IgG1 antibodies against most of the tested antigens including CL, PE, dsDNA, collagen and TNP were found in the kidney eluate of mercury- but not of saline-injected (NZB×NZW)F$_1$ mice (Fig. 4a). However, acid eluate of mercury-injected mice showed no reactivity against nucleolar antigens (ANolA) (Fig. 4b).

Since kidney eluates from mercury-injected (NZB×NZW)F$_1$ mice showed a significant increase in levels of IgG2a antibodies (Fig. 3b), the specificity of this isotype present in the kidney eluates was also measured. A slight increase in levels of IgG2a antibodies against PE, CL, dsDNA and collagen but not against nucleolar antigen was found in the kidney eluate of mercury-injected (NZB×NZW)F$_1$ mice compared with that of saline-injected controls (not shown). Taken together, results from this and our previous study [6] clearly demonstrate a consistent correlation between circulating autoantibodies and renal deposited autoantibodies in (NZB×NZW)F$_1$ hybrids after injection with mercury.

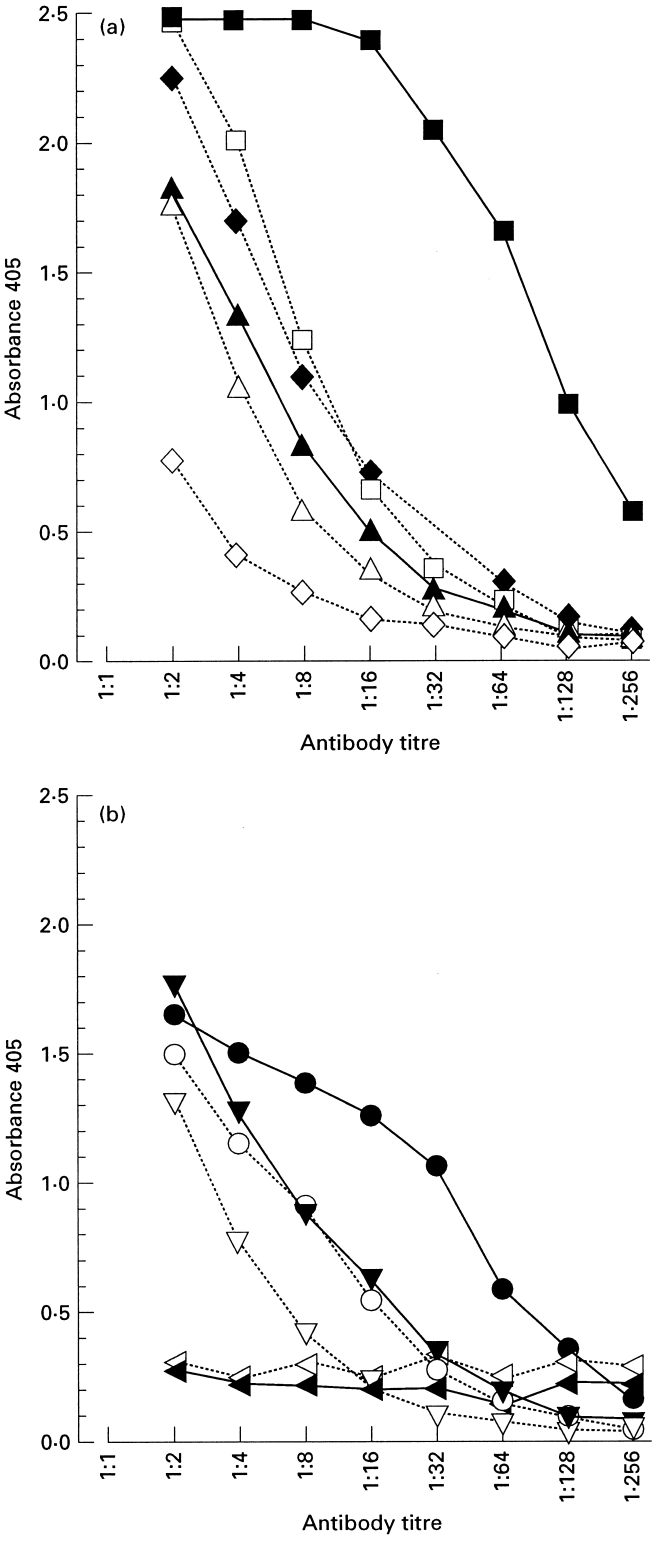

Fig. 3. Kidney eluate of mercury-injected young (NZB×NZW)F$_1$ mice contains antibodies of various isotypes. Deposited antibodies in the kidneys of same experimental mice as described in Fig. 2 were eluted using an acid buffer method. Presence of IgM (▼, ▽), IgG1 (■, □), IgG2a (●, ○), IgG2b (▲, △), IgG3 (◆, ◇) and IgE (◀, ◁) antibodies in the kidney eluates of young (NZB×NZW)F$_1$ mice treated with either mercury (solid symbols) or saline (open symbols) was measured by ELISA.

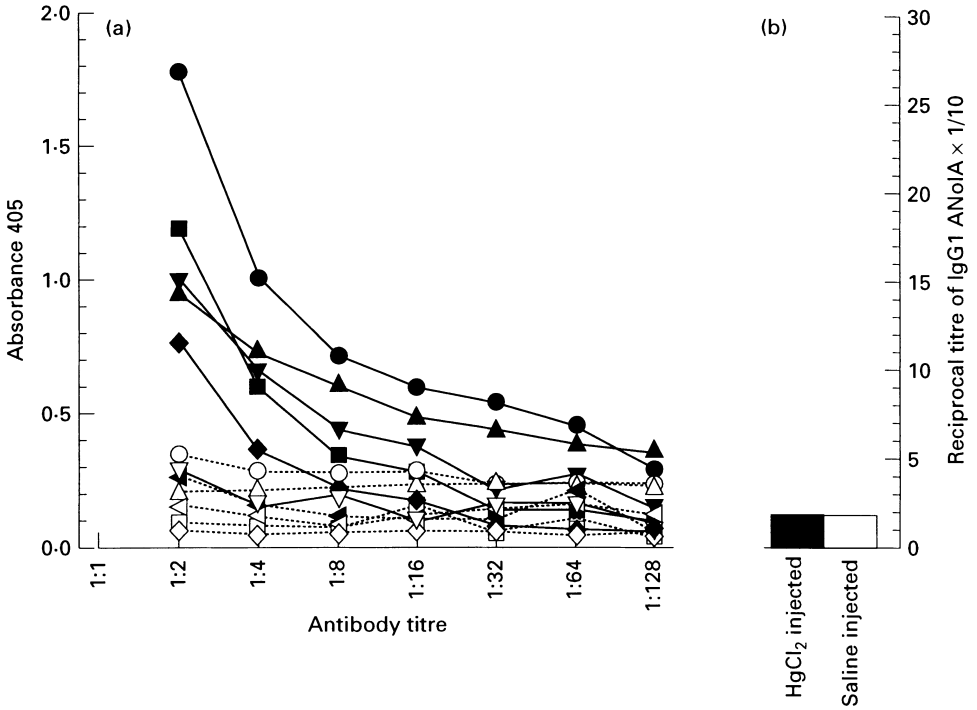

Fig. 4. IgG1 antibodies in the kidney eluates of mercury-injected young (NZB × NZW)F_1 mice are directed against different antigens. Kidney eluates of mercury- (solid symbols) and saline-injected mice (open symbols) were tested for the presence of IgG1 antibodies against phosphatidylethanolamine (▲, △), cardiolipin (●, ○), dsDNA (■, □), collagen (◆, ◇), trinitrophenol (TNP) (▼, ▽), thyroglobulin (▲, △) and nucleolar antigen (ANolA) by using ELISA (a) and indirect immunofluorescence (b).

Proteinuria

To test whether renal deposits of autoantibodies in mercury-injected (NZB × NZW)F_1 mice cause tissue damage in the kidneys, urinary protein excretion in the same experimental mice as described in Fig. 4 was measured using standard dipsticks. As shown in Table 1, no significant difference was found between mercury- and saline-injected mice with regard to urinary protein concentration.

DISCUSSION

In the present study, we found that injection of mercury into young (NZB × NZW)F_1 mice induced a high increase in levels of all immunoglobulin isotypes (except for IgG2b). High titres of granular deposits of IgM, IgG1, IgG2a, IgG3 were also found in kidneys of mecury-injected (NZB × NZW)F_1 mice. The dominant mercury-induced renal deposited antibody was of IgG1 isotype and had several specificities, which correlated with the specificities of circulating IgG1 antibodies [6]. In addition, we showed that the mercury-induced renal deposits of immunoglobulins did not lead to the development of proteinuria in young (NZB × NZW)F_1 mice.

Table 1. Proteinuria analysis in mercury- and saline-injected young (NZB × NZW)F_1 mice

Treatment*	Number of mice	Mean value of urinary protein concentration*
$HgCl_2$	5	⩽30 mg/100 ml
Saline	5	⩽30 mg/100 ml

* Young (NZB × NZW)F_1 hybrids were continually injected with either subtoxic doses of $HgCl_2$ or saline for 6 weeks, and at the end of experiment the proteinuria was assayed with a standard dipstick.

Several studies have shown that in mercury-susceptible H-2^s mice, upon exposure to mercury, increased production of IgG1 and IgE antibodies was accompanied by increases in the synthesis of other immunoglobulin isotypes (IgG2b and IgG2a) as well [7,10]. We also found that injection with mercury in (NZB × NZW)F_1 mice induced an increase in the production of all immunoglobulin isotypes except for IgG2b antibodies. The exact mechanism(s) by which mercury activates B cells leading to antibody production of different isotypes is not understood. However, since it is known that mercury-induced B cell activation is T helper (Th) cell-dependent [1–3], it is conceivable that cytokines derived from Th cells play an important role. Indeed, it has been demonstrated that synthesis of high levels of a so called Th2-derived cytokine, IL-4, was responsible for mercury-induced IgG1 and IgE antibody production [10,11]. Production of high levels IgG2a and IgG3 in mercury-injected young (NZB × NZW)F_1 mice suggests that in addition to IL-4, other cytokines such as interferon-gamma (IFN-γ), which is considered as a switch factor for these immunoglobulin isotypes [12], is also involved. Studies are in progress to test this suggestion.

The hallmark of mercury-induced autoimmune manifestations in rodents is the development of renal immune complex deposits [1–3]. In highly mercury-susceptible rats, mercury-induced renal

immune complex deposition was found to be biphasic (reviewed in [13]). The first phase, which appeared 2–3 weeks after the start of mercury administration, was characterized by the linear binding of IgG anti-glomerular basement membrane (anti-GBM) autoantibodies along the GBM [13]. Continual injection with mercury led to appearance of the second phase in which the linear binding of IgG along the GBM was replaced by granular immune complex deposits along the outer side of the GBM [13]. In contrast, several studies have demonstrated that mercury-induced renal immune complex deposits in susceptible mice, which developed 3 weeks after initiation of the mercury injection, was monophasic and appeared in a mesangial granular pattern [4,5,7,14]. We also found that injection of mercury into young (NZB×NZW)F$_1$ resulted in the development of early monophasic immunoglobulin deposits in the glomeruli with mesangial granular distribution. The reason that mercury-susceptible mice develop granular but not linear renal immune complex deposits is possibly that they do not produce anti-GBM autoantibodies after injection with mercury.

Mercury-induced renal immune complex deposits in highly susceptible mouse strains such as SJL and (NZB×NZW)F$_1$ share several similarities. For instance, as discussed above, both strains develop renal immunoglobulin deposits in a granular distribution after mercury injection ([6,7,14] and this study). Moreover, in both strains the isotype of the deposited antibodies in the kidney correlate with the increased levels of circulating antibodies (same isotypes) ([6,7] and this study). However, these two mouse strains show a major difference in the specificity of the antibodies in the kidney eluates. Antibodies eluted from the kidneys of mercury-injected SJL mice were found be directed mainly against nucleolar antigens [14]. In contrast, acid-eluate from the kidneys of mercury-injected (NZB×NZW)F$_1$ mice showed reactivity against several different antigens, except against nucleolar antigens. Taken together, these findings indicate that in mercury-susceptible mice, injection with mercury will lead to renal deposition of antibodies/autoantibodies regardless of their specificities. Whether the mercury-induced renal immune complex deposits are formed by accumulation of circulating immune complexes in the kidney or by direct binding of antibodies/autoantibodies to the self-components that are trapped in the glomerular capillary wall or by direct binding of cross-reactive/polyreactive antibodies to glomeruli, remains to be determined.

A striking finding in the present study was that the deposition of high titres of immunoglobulins in the glomeruli of mercury-injected young (NZB×NZW)F$_1$ mice did not result in kidney injury as determined by proteinuria. Interestingly, chronic injection with mercury into other mercury-susceptible mouse strains such as SJL mice was found not to cause severe glomerulonephritis either [7,14]. Since mercury-injected SJL mice exhibited a modest renal deposition of C3, and since mainly IgG1 antibodies formed deposits in the kidneys of these mice [7], it has been proposed that the weak complement activation and the deposition of mainly non-complement fixing IgG1 antibodies are responsible for the lack of kidney damage [7]. The finding that mercury- and saline-injected young (NZB×NZW)F$_1$ mice had similar levels of renal deposited C3, and that in mercury-injected young (NZB×NZW)F$_1$ mice the dominant renal deposited antibody was of IgG1 isotype, indicate that the same proposition might be true for the lack of kidney failure in these mice.

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