Xenobiotic Acceleration of Idiopathic Systemic Autoimmunity in Lupus-Prone BXSB Mice

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The diverse genetic backgrounds of lupus-prone murine models, which produce both quantitative and qualitative differences in disease expression, may be a valuable resource for studying the influence of environmental exposure on autoimmune disease in sensitive populations. We tested this premise by exposing autoimmune-prone BXSB and the nonautoimmune C57BL/6 mice to the heavy metal mercury. Although both strains express a nonsusceptible H-2 haplotype, exposure to mercury accelerated systemic autoimmunity in both male and female BXSB mice, whereas the C57BL/6 mice were resistant. The subclasses of antichromatin antibodies elicited in BXSB mice by mercury exposure were more consistent with the predominant Th1-type response of idiopathic disease than with the Th2-type response found in mercury-induced autoimmunity (HgIA). The appearance and magnitude of both humoral and cellular features of systemic autoimmunity correlated with the mercury dose. Furthermore, environmentally relevant tissue levels of mercury were associated with exacerbated systemic autoimmunity. These studies demonstrate that xenobiotic exposure can accelerate spontaneous systemic autoimmunity, and they support the possibility that low-level xenobiotic exposure enhances susceptibility to systemic autoimmunity in genetically susceptible individuals. *Key words*: autoantibodies, autoimmunity, *in vivo* animal models, lupus, rodent. *Environ Health Perspect* 109:27–33 (2001). [Online 30 November 2000]
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Autoimmune diseases are associated with numerous immunologic and pathologic abnormalities, but the factors critical for inducing disease are poorly understood (*1*,*2*). Although genetic predisposition may be a prerequisite for the development of spontaneous systemic autoimmune diseases, such as systemic lupus erythematosus (SLE) (*3*), the incomplete concordance in SLE twin studies (*4*) suggests that exogenous or environmental factors are also important. Some of the best examples of systemic autoimmune disease triggered by environmental agents come from chemical-induced autoimmunity, and have been described both in humans (*5*,*6*) and in animal models (*5*,*7*,*8*). In addition, studies using murine models of lupus have shown that a variety of other exogenous agents can accelerate the onset of autoimmunity in genetically predisposed hosts (*9*–*12*); however, no specific factor has been documented to play a vital role in idiopathic SLE.

Delineation of the role that environmental agents play in accelerating and/or exacerbating human autoimmune disease generally has not considered the fact that a subset of individuals may be particularly sensitive because of genetic predisposition. Although associations between environmental exposure and certain autoimmune diseases have been identified in epidemiologic studies (*6*,*13*), these studies have not identified those at risk. In addition, the genes that predispose to autoimmunity and their responses to environmental exposures have yet to be determined. Therefore, it has not been possible to test in humans whether genetic predisposition to spontaneous autoimmunity increases the sensitivity to environmental agents. The availability of animal models that spontaneously develop systemic autoimmunity provides an alternative approach to studying this question. Moreover, due to derivation from different genetic backgrounds, murine models of SLE manifest both quantitative and qualitative differences in disease expression that can be exploited (*14*,*15*).

Certain heavy metals, such as mercury, are potent environmental agents toxic to the immune system which can provoke not only immunosuppressive but also immunostimulatory effects in many species, including humans and rodents (reviewed in *8* and *16*). Studies with nonautoimmune-prone animal models suggest that the immunoactivating properties of mercury can be divided into three major pathologic sequelae: lymphoproliferation, hypergammaglobulinemia, and the development of systemic autoimmunity manifested as production of autoantibody and immune-complex disease (*8*,*15*). Elicitation of these pathologic features depends on genetic background, with lymphadenopathy occurring in most strains, whereas autoimmunity—which in nonautoimmune-prone strains is controlled largely by the MHC gene—is more restricted (*17*,*18*). Exposure to mercury of autoimmune-prone MRL-+/+ and NZBWF1 mice accelerates autoimmunity in a strain-specific manner, with the most severe manifestations occurring in the NZBWF1 (*19*). Contrastingly, MRL-*lpr* mice, which are deficient for the Fas apoptosis-promoting gene and manifest more severe disease, exhibited little acceleration of humoral autoimmunity, suggesting that strains with highly accelerated disease may be less sensitive to environmental exposure. In addition, comparison with the H-2 compatible nonautoimmune-prone AKR mice indicated that both MHC and non-MHC genes contributed to acceleration of disease expression in the MRL-+/+. In these studies, lupus-prone strains expressed MHC haplotypes that predispose to HgIA. Therefore, the extent to which susceptibility was due to HgIA or to underlying non-MHC susceptibility genes could not be clearly delineated.

In this study, we examined the influence of mercury exposure and dosage on expression of autoimmunity in the lupus-prone BXSB mice. In this strain, severe accelerated autoimmunity with early mortality occurs in males due to the Y-chromosome linked gene *Yaa*, whereas females develop a delayed and much milder form of disease (*3*,*15*). Importantly, the MHC haplotype of this strain ($H\text{-}2^b$) is considered resistant to $HgCl_2$ since other strains with this haplotype, including the C57BL/6, which was used as the control in this study, are much less susceptible to HgIA than mice of otherwise similar backgrounds expressing s, k, or d haplotypes (*8*,*17*,*18*). Mercury exposure accelerated autoimmunity in BXSB mice consistent with idiopathic rather than HgIA disease based on the predominant types of

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IgG autoantibody subclasses detected. Dose–response studies suggested that environmentally relevant tissue levels of mercury were able to exacerbate systemic autoimmunity. These studies support the concept that low-level xenobiotic exposure can accelerate idiopathic systemic autoimmunity in genetically susceptible hosts.

Materials and Methods

Mice. Male and female BXSB (H-2^b), and female C57BL/6 (H-2^b) mice were obtained from The Scripps Research Institute Animal Colony (La Jolla, CA) and maintained under specific pathogen-free conditions. All experimental procedures using animals followed the guidelines set down in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (1996).

Treatment of mice. In short-term exposure studies, groups of up to eight 4-week-old mice were injected subcutaneously (sc) twice per week for 4 weeks with 100 µL PBS containing 40 µg $HgCl_2$, or PBS alone. Mice were bled for sera before the first injection and at sacrifice on day 30. Autopsies were performed as described previously (*20*). Samples of kidney and spleen were taken for analysis of immune-complex deposition as described previously (see below).

Separate experiments were performed to examine for the persistence of autoimmunity following mercury exposure. Eight-week-old female BXSB and C57BL/6 mice were treated for 4 weeks with either $HgCl_2$ or PBS and then bled at 2-week intervals to assess autoantibody levels. At sacrifice, samples of kidney and spleen were obtained and proteinuria was determined.

To examine the effect of $HgCl_2$ dose on acceleration of autoimmunity, we injected groups of 8-week-old female BXSB mice with 40, 4, 0.4, or 0.04 µg $HgCl_2$ in PBS twice per week for 11 months. Control mice received PBS. Mice were monitored for disease as above. Samples of kidney were also analyzed for mercury content as described previously (*21*).

Detection of serum antibodies. ANA was detected as described previously (*22*) using HEp-2 cell slides (Bion Enterprises, Park Ridge, IL). Sera were diluted 100-fold in PBS containing 0.5% bovine serum albumin (BSA), 0.1% BGG, 0.001% gelatin, and 0.05% Tween 20 before assay. Goat anti-mouse IgG-FITC (Caltag Laboratories, San Francisco, CA), diluted 100-fold in PBS containing 0.5% BGG, 0.1% BSA, and 0.05% Tween 20, was used as detecting reagent. Antifibrillarin (nucleolar) monoclonal antibody 72B9 (*23*) was used as positive control.

Antichromatin antibodies were detected by ELISA (*24*). Sera were diluted 100-fold before assay, and chromatin-bound antibodies were detected with HRP-conjugated goat anti-mouse IgG (Caltag Laboratories), diluted 2,000-fold. Antichromatin monoclonal antibody 1D12 (*25*) was used as positive control. The IgG subclass of antichromatin antibodies in female BXSB mice was also determined by ELISA, using serum dilutions of 1/100–1/400 and a saturating (1/1,000) dilution of subclass detecting reagent. Horseradish peroxidase (HRP)-conjugated anti-IgG1 and IgG2b were from Caltag Laboratories, anti-IgG2a^{a+b} was from Pharmingen (San Diego, CA), and anti-IgG3 was from Southern Biotechnology Associates (Birmingham, AL).

Serum immunoglobulin quantitation. Serum IgG, IgG1, and IgG2a levels were quantified by ELISA (*26,27*). ELISA plates were coated with 200 µL 2 µg/mL goat anti-mouse kappa light chain antibody (Caltag Laboratories) diluted in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Plates were postcoated for 1 hr with 0.1% gelatin in PBS followed by 3 washes with PBS-0.05% Tween 20. Sera were diluted in serum diluent (*26*). A standard curve was generated by serial dilutions of polyclonal mouse reference serum containing predetermined levels of Ig isotypes (The Binding Site, Birmingham, UK). Diluted sera were incubated in duplicate while shaking for 2.5 hr followed by 3 washes with PBS-0.05% Tween 20. HRP-conjugated goat anti-mouse IgG, or IgG1 antibodies (Caltag Laboratories) were diluted in anti-Ig diluent (*26*) and incubated with shaking for 90 min. After 3 washes with PBS-0.05% Tween 20 and 4 washes with PBS, ABTS substrate solution was added and the optical density (OD) read at 405 nm. Determination of IgG2a in sera from BXSB and C57BL/6 mice required use of reagents specific for the b allotype of IgG2a (*27*). Serum IgG, IgG1, and IgG2a concentrations were calculated by extrapolation from the linear portion of standard curves.

Tissue immune-complex deposits. A 2–3 mm thick section of the kidney and spleen were snap-frozen in isopentane-CO_2 and examined by direct immunofluorescence as described previously (*28*). Briefly, 4–5 µm thick cryostat sections were fixed in ethanol and incubated with doubling dilutions of FITC-conjugated goat antibodies to IgG (gamma chain specific) and C3 (Southern Biotechnology Associates). The end-point titer of the immune deposits was defined as the highest dilution of antibody at which specific fluorescence could be detected. The presence of granular deposits in small and medium-sized arteries was also examined. The slides were examined under blinded conditions.

Light microscopy. A 2–3 mm thick section of the kidney was immersed in Histochoice (Amresco, Solon, OH), and embedded in paraplast, and 1–2 µm sections were cut. The sections were stained with periodic acid–Schiff (PAS) reagent and with periodic-acid-silver-methenamine. The types of glomerular pathology were determined, and the degree of endocapillary cell hyperplasia was scored for each animal as follows: 0 = normal; 0.5 = just detectable alteration; 1 = slight; 2 = moderate; 3 = strong; and 4 = maximal. Slides were examined without knowledge of treatment or other results.

Urinary protein. Proteinuria was measured by Chemstrip 2 GP test strips as described by the manufacturer (Boehringer Mannheim Diagnostics, Indianapolis, IN). To compare results between groups, the milligram protein per deciliter scale was graded (0 = negative, 1 = trace, 2 = 30, 3 = 100, 4 = 500 mg/dL). Intermediate values were graded at 0.5 units above the lower value (i.e., 1.5 = trace to 30 mg/dL). In the mercury dose–response study, proteinuria was measured using the Bradford assay (Pierce, Rockford, IL) with BSA as the protein standard.

Flow cytometry analysis of peripheral blood lymphocytes (PBL). Flow cytometry procedures were performed as described previously (*29*). Briefly, PBL were stained with the following antibodies (Pharmingen, La Jolla, CA): APC-conjugated anti-mouse CD11b, FITC-conjugated anti-CD3e, cychrome-conjugated anti-CD45R/B220, and PE-conjugated anti-I-A^b antibodies. The anti-CD16/CD32 (FcγIII/IIR) antibody, 2.4G2, was also added to block nonspecific FcR binding. Data were acquired on the FACSVantageTMSE and analyzed using CELLQuest (Becton-Dickinson, Sunnyvale, CA). Ten to twenty thousand events were collected, and live-gated cells, based on forward and side scatter characteristics, were examined.

Statistical analysis. Unless otherwise noted, all data are expressed as mean ± 1SD. Groups were compared by unpaired *t*-test, single-factor analysis of variance, Mann-Whitney *U* test, or Fisher's Exact Test as appropriate. Comparisons are of $HgCl_2$-treated mice with PBS-treated animals; $p < 0.05$ was considered significant.

Results

Effects of short-term mercury exposure on 4-week-old male BXSB mice. Although male BXSB mice are highly susceptible to SLE, 4 weeks of mercury exposure significantly elevated levels of serum IgG and IgG1 and IgG2a subclasses, compared to PBS control animals (Table 1). Antibodies to nuclear antigens (ANA) consisting of a dense fine to

homogeneous nuclear speckling of interphase cells and metaphase chromosomes was found in 88% of male mice exposed to $HgCl_2$, while pretreatment bleeds as well as PBS-treated mice had less frequent ANA responses (range 0–25%). Similarly, levels of antichromatin antibodies in $HgCl_2$-exposed male mice were elevated above those found in the PBS group (Table 1).

When compared to PBS-treated animals, $HgCl_2$-treated male BXSB mice had increased organ wet weight for spleen and the draining cervical lymph nodes but not the mesenteric lymph nodes (Table 2). Glomerular deposits, localized to the mesangium, were observed in both $HgCl_2$-exposed and PBS-control animals, with mean titers higher in the mercury group (Table 2). This, however, did not reach statistical significance because of large variations in individual titers. Nevertheless, compared to PBS controls, mercury-exposed male BXSB mice showed significant increases in endocapillary cells as observed by light microscopy (1.62 ± 0.44 vs. 0.56 ± 0.050; $p < 0.01$) (Figure 1). Glomerular basement membranes were normal and there was no inflammation. Interestingly, although non-glomerular deposits to the kidney vessel wall are typically seen in HgIA, they were absent in the mercury-exposed male BXSB mice. This suggests that the systemic autoimmunity induced in male BXSB mice by $HgCl_2$ was more consistent with spontaneous lupus than with HgIA.

Effects of short-term mercury exposure on 4-week-old female BXSB mice. Female BXSB mice are considerably less susceptible to SLE than their male counterparts, and develop mild SLE in late life. Nonetheless, mercury-exposed female BXSB mice also had elevations of serum IgG, IgG1, and IgG2a compared to PBS controls, although levels were lower than those in male $HgCl_2$-treated mice (Table 1). Similar to male mice, 88% of females exposed to $HgCl_2$ developed ANAs that consisted of dense fine to homogeneous nuclear speckling of interphase cells and metaphase chromosomes (Table 1). PBS-treated mice, as well as all pretreatment bleeds, had less frequent ANA responses (range 0–29%). Although two $HgCl_2$-exposed female mice had elevated antichromatin antibodies, the response of this group was not statistically different from the PBS-treated group (Table 1).

$HgCl_2$-treated female BXSB mice had greater organ wet weight for spleen and cervical lymph nodes but not mesenteric lymph nodes, compared to PBS treated animals (Table 2). The two $HgCl_2$-treated female mice with elevated antichromatin antibodies also had low titers of IgG deposits in the mesangium, whereas the remaining six $HgCl_2$-treated mice as well as all mice given PBS showed no IgG deposits (Table 2). The titers of glomerular C3 deposits were similar between the two groups. Histologic examination revealed increased glomerular endocapillary cells in $HgCl_2$-treated mice (2.38 ± 0.92 vs. 1.29 ± 0.57, $p < 0.05$), but basement membranes were normal and there was no inflammation. Vessel wall deposits were not found in the kidney or spleen in either group.

Effect of short-term mercury exposure on nonautoimmune-prone 4-week-old female C57BL/6 mice. Compared to PBS-treated mice, mercury-exposed C57BL/6 mice developed hypergammaglobulinemia with

Table 1. Immunoglobulin levels and autoantibodies in BXSB mice following $HgCl_2$ exposure.[a,b]

			Immunoglobulin level				
Sex	No.	Treatment	IgG (mg/mL)	IgG1 (mg/mL)	IgG2a (μg/mL)	ANA (pos/no.)	Antichromatin Ab
Male	8	Pre PBS	2.5 ± 1.3	0.03 ± 0.10	77 ± 59	1/8	0.00 ± 0.00
		Post	4.7 ± 3.6	0.28 ± 0.16	126 ± 60	1/8	0.26 ± 0.31
	8	Pre $HgCl_2$	1.9 ± 0.9	0.20 ± 0.11	58 ± 36	0/8	0.13 ± 0.19
		Post	13.9 ± 3.0##	3.34 ± 0.59##	802 ± 133##	7/8	0.98 ± 0.57**
Female	8	Pre PBS	1.9 ± 2.0	0.19 ± 0.08	17 ± 6	1/7	0.01 ± 0.01
		Post	5.2 ± 2.6	0.24 ± 0.05	34 ± 14	2/7	0.03 ± 0.03
	8	Pre $HgCl_2$	1.5 ± 0.7	0.13 ± 0.06	10 ± 9	1/8	0.12 ± 0.51
		Post	6.7 ± 1.8*	2.17 ± 0.42##	96 ± 45#	7/8	0.60 ± 0.93

Abbreviation: pos/no., number positive/total number. *p*-Values are from comparison of mercury-treated groups with PBS-treated group.
[a]Mice were 4 weeks old at the beginning of the experiment. [b]Values are given as mean ± 1SD. *$p < 0.05$. **$p < 0.01$. #$p < 0.005$. ##$p < 0.0001$.

Table 2. Pathologic changes in BXSB mice following $HgCl_2$ exposure.[a]

			Organ wet weight (mg)			Kidney immunopathology[b,c]			
				Cervical	Mesenteric	Glomerular		Vessel	
Sex	No.	Treatment	Spleen	LN	LN	IgC	C3	IgG	C3
Male	8	PBS	116 ± 5	24 ± 3	47 ± 1	151	830	0	0
	8	$HgCl_2$	237 ± 27†	108 ± 13##	61 ± 9	2,348	3,620	0	0
Female	7	PBS	62 ± 3	32 ± 3	52 ± 6	0	476	0	0
	8	$HgCl_2$	79 ± 3[d]#	49 ± 3##	47 ± 2	1	453	0	0

LN, lymph node. *p*-Values are from comparison of mercury-treated groups with PBS-treated group.
[a]Mice were 4 weeks old at the beginning of the experiment. [b]Immunopathology data is given as geometric mean. [c]Data are expressed as the reciprocal titer. [d]Data from seven animals. †$p < 0.001$. #$p < 0.005$. ##$p < 0.0001$.

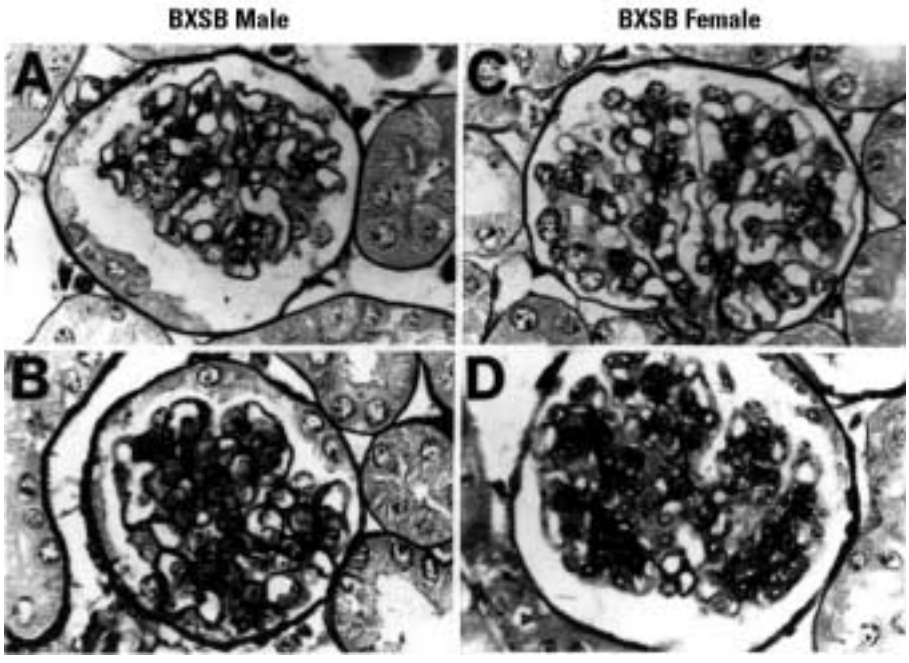

Figure 1. Representative glomerular light microscopy of male and female BXSB mice. Four-week-old mice were treated with PBS (*A, C*) or $HgCl_2$ (*B, D*) for 4 weeks. Endocapillary cell proliferation was scored as described in "Materials and Methods." The kidney in (*A*) was scored as 0 and (*B*) as 2+. The kidney in (*C*) was scored as 1+ and (*D*) as 3+. Magnification x800.

elevations in IgG, IgG1, and IgG2a and a dense fine speckled ANA pattern (Table 3). However, the mean antichromatin antibody level was not elevated (Table 3). One PBS-treated mouse had an elevated antichromatin response (OD405 = 3.1), suggesting a low penetrance susceptibility to antichromatin autoantibody production in this strain. None of the mercury-treated C57BL/6 mice had increased deposition of immunoglobulin or complement (C3) in the kidney or spleen, and histology by light microscopy was unremarkable (data not shown). The lack of elevated antichromatin antibodies and kidney pathology in mercury-exposed C57BL/6 mice indicates that non-MHC genes are the major contributors to the $HgCl_2$-induced responses in BXSB mice.

Transient $HgCl_2$ exposure in 8-week-old female BXSB mice. To study the long-term effects of $HgCl_2$ following transient exposure, we gave 8-week-old female BXSB mice $HgCl_2$ or PBS for 1 month and then followed them for 32 weeks without further treatment. Mice were tested for autoantibodies at 2-week intervals and examined for immunopathology at the end of the experiment. C57BL/6 mice were treated similarly and tested only for autoantibodies because previous studies have already established that immunohistologic abnormalities do not develop in this strain [see above (*17*,*18*)].

The antichromatin response in $HgCl_2$-treated BXSB mice was significantly elevated above that of PBS-treated mice up to 14 weeks after treatment (Figure 2), following which PBS-treated mice began to develop antichromatin antibodies. In contrast, $HgCl_2$-treated C57BL/6 mice had low levels of antichromatin Ab, not significantly different from their PBS controls (data not shown). Compared to 4-week-old female BXSB (Table 1), older 8-week-old female mice were more susceptible to $HgCl_2$-induced acceleration of antichromatin antibodies.

Histologic examination of kidneys revealed titers of glomerular IgG and C3 deposits that were higher in $HgCl_2$-treated mice but varied widely among individual animals (Table 4). Three of the control mice showed a combination of granular capillary wall and mesangial IgG staining, whereas the remaining four had only mesangial staining. Of the $HgCl_2$-treated mice, one showed a combined granular capillary wall and mesangial IgG staining, two showed only granular capillary wall staining, and the remaining five mice had only mesangial staining. Mice with staining of the capillary loops, with or without concomitant mesangial staining, showed endocapillary cell hyperplasia. Most animals also had a moderate thickening and irregularity of the glomerular basement membrane. Mesangial deposits were associated only with slight endocapillary cell hyperplasia. Four of the $HgCl_2$-treated but none of the PBS-treated mice had deposits of IgG in kidney vessels. The titer and frequency of immunoglobulin and complement deposits in the spleen was also higher in $HgCl_2$-treated mice, with seven of eight having IgG deposits and six of those seven having C3 deposits, whereas only three PBS mice had low titer deposits of IgG and C3 in splenic vessels. The increased titers and frequency of immune reactants in the tissues of $HgCl_2$-treated mice pointed to the development of more severe disease in these animals compared to PBS-treated mice. Consistent with this was the finding that three of the seven $HgCl_2$-treated mice tested had > 30 mg/dL protein in their urine, whereas all five PBS mice tested had ≤ 30 mg/dL ($p < 0.05$; Table 4). Thus, compared to PBS controls,

Table 3. Immunoglobulin levels and autoantibodies in female C57BL/6 mice following $HgCl_2$ exposure.[a,b]

		Immunoglobulin level				
No.	Treatment	IgG (mg/mL)	IgG1 (mg/mL)	IgG2a (μg/mL)	ANA (pos/no.)	Antichromatin Ab
8	Pre PBS	1.07 ± 0.37	0.15 ± 0.09	4.2 ± 2.1	0/8	0.00 ± 0.00
	Post	2.77 ± 0.71	0.06 ± 0.42	13.6 ± 15.8	1/8	0.40 ± 1.11
8	Pre $HgCl_2$	1.49 ± 0.51	0.28 ± 0.26	3.2 ± 1.8	0/8	0.00 ± 0.00
	Post	7.68 ± 1.69§	1.62 ± 0.33†	73.5 ± 35.4†	8/8	0.17 ± 0.23

Abbreviation: pos/no., number positive/total number. *p*-Values are from comparison of mercury-treated groups with PBS-treated group.
[a]Mice were 4 weeks old at the beginning of the experiment. [b]Values are given as mean ± 1SD. §$p < 0.02$. †$p < 0.001$.

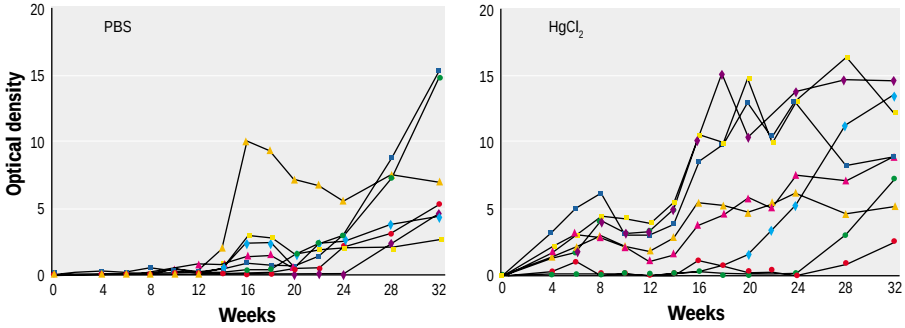

Figure 2. Comparison of the magnitude and persistence of antichromatin antibodies in BXSB after exposure to mercury. Female mice were injected with either $HgCl_2$ or PBS for 4 weeks and then bled at 2-week intervals. Serum antichromatin antibodies were determined by ELISA. Different symbols denote individual mice. Significant differences were found between the two groups of BXSB mice at 4 ($p < 0.01$), 6 ($p < 0.005$), 8 ($p < 0.01$), 10 ($p < 0.01$), 12 ($p < 0.025$), and 14 ($p < 0.038$) weeks.

Table 4. Pathologic changes in 40-week-old female BXSB mice following $HgCl_2$ exposure.

		Kidney immunopathology[a]				Spleen immunopathology[a]		
		Glomerular		Vessel		Vessel		
No.	Treatment	IgG	C3	IgG	C3	IgG	C3	Urinary protein
7[b]	PBS	263	707	0	0	3	2	1.3 ± 0.5[c]
8	$HgCl_2$	293	3,044	5	1	180§	43	2.4 ± 1.0*[d]

p-Values are from comparison of mercury-treated groups with PBS-treated groups.
[a]Immunopathology data are given as geometric mean, and data are expressed as the reciprocal titer. [b]One animal died during bleeding. [c]Data from five animals. [d]Data from seven animals.*$p < 0.05$. §$p < 0.02$.

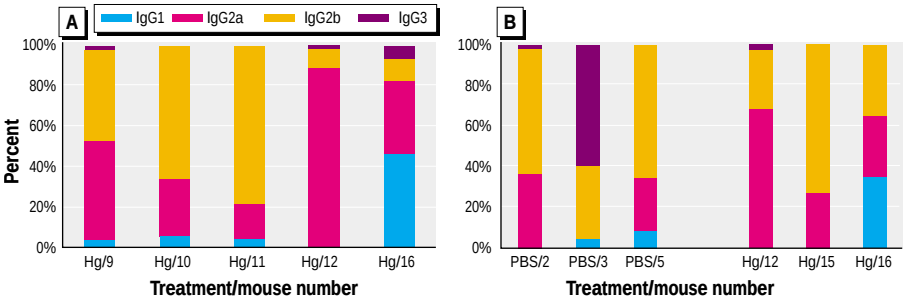

Figure 3. IgG subclass of antichromatin antibodies in BXSB mice with spontaneous and $HgCl_2$-accelerated autoimmunity. Sera from mice at 4–6 (*A*) or 32 (*B*) weeks after the start of $HgCl_2$ or PBS treatment were analyzed for IgG subclass antichromatin antibodies by ELISA. The percent contribution of each subclass was determined from the sum of the OD405 values for the individual subclasses.

$HgCl_2$-treated mice have more severe disease long after termination of exposure.

IgG subclasses of antichromatin antibodies in BXSB mice with idiopathic and xenobiotic accelerated autoimmunity. HgIA is associated with a Th2-like response characterized by increases predominantly in autoantibodies of the IgG1 subclass (*30*). $HgCl_2$ also has been shown to promote deviations from Th1 to Th2 predominance in several autoimmune diseases (*27*). In contrast, the spontaneous antichromatin response in BXSB mice is mainly of the IgG2a and IgG2b subclasses (*31*) due to the predominant Th1-like cytokine response in this strain (*32*). Thus, the IgG isotype autoAb levels of mercury-treated BXSB mice might indicate whether the accelerated autoimmunity resulted from the spontaneous (Th1) or HgIA (Th2) types of response. Sera from the long-term study of female BXSB mice treated transiently with $HgCl_2$ were examined for antichromatin IgG subclass levels. Immediately after 4 weeks of mercury exposure, all five antichromatin antibody-positive mice had subclasses that were predominantly of the IgG2a and IgG2b isotypes (Figure 3). Except for an IgG1 response in one mouse (No. 16), there were few antichromatin antibodies of the IgG1 and IgG3 subclasses. Thirty-two weeks after treatment began, the antichromatin IgG subclasses in PBS- and $HgCl_2$-treated mice were very similar, consisting in particular of IgG2b as well as IgG2a subclasses. One PBS mouse (No. 3) had a strong IgG3 response, while $HgCl_2$-treated mouse No. 16 had retained its IgG1 and IgG2a responses and increased its IgG2b response (Figure 3).

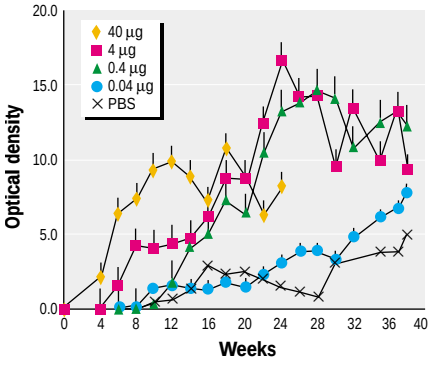

Figure 4. Effect of $HgCl_2$ dose on antichromatin antibodies in female BXSB mice. Female mice were injected with 40, 4, 0.4 or 0.04 µg $HgCl_2$ in PBS or with PBS alone for the length of the experiment. Mice were bled at 2-week intervals and serum antichromatin antibodies determined by ELISA. Data are expressed as mean ± SE for each treatment group.

Effects of long-term exposure and lower doses of $HgCl_2$ on the development of autoimmunity in female BXSB mice. Although the dose of $HgCl_2$ used in the above studies could accelerate autoimmunity in BXSB mice, the resulting tissue levels of mercury would be greater than that found in humans exposed to environmental levels of mercury (*21*,*33*,*34*). To determine whether lower levels of $HgCl_2$ exposure could also trigger autoimmune disease in female BXSB mice, we gave groups of 8-week-old animals either 40, 4, 0.4, or 0.04 µg $HgCl_2$ twice per week for almost 11 months. For mice given the highest dose of 40 µg, all succumbed or were moribund by 24 weeks of treatment after becoming severely cachexic. One mouse each in the 4 µg and 0.4 µg groups died at 31 and 38 weeks, respectively. Survival of mice in the 0.04 µg and PBS groups was not affected by treatment.

The antichromatin Ab response of female BXSB mice was clearly induced by exposure to $HgCl_2$ in a dose-dependent manner even with doses as low as 0.4 µg. Elevated levels were detected first in the 40 µg $HgCl_2$ group as soon as 4 weeks after the start of treatment and then 4 weeks later in the 4 µg $HgCl_2$ group (Figure 4). After 16–20 weeks of treatment, the 0.4 µg $HgCl_2$ group developed high levels of antichromatin Ab, equivalent to those in the 40 and 4 µg $HgCl_2$ groups. Antichromatin Ab levels subsequently remained elevated in these three groups for the remainder of the experiment. Even the lowest 0.04 µg $HgCl_2$ group tended to have higher antichromatin Ab levels than the PBS control at the end of the treatment period, but the levels did not reach those of groups given higher doses.

The accelerated autoimmune disease in male BXSB mice is associated with increases in memory/effector phenotype T cells and Mac-1 positive macrophages in the peripheral blood that may play a role in disease pathogenesis (*14*). When peripheral blood cells were examined after 32 weeks of $HgCl_2$ exposure, no changes were found in the percentage of peripheral blood mononuclear cell (PBMC) $CD4^+$ T cells or B ($B220^+$) cells (Table 5). However, mice receiving 4 µg $HgCl_2$ had increases in activated phenotype ($CD44^{hi}$) $CD4^+$ T cells ($p < 0.005$) and an increase in percentage of CD62L (Mel-14)^{lo} $CD4^+$ T cells, particularly when compared to the 0.04 µg $HgCl_2$ group ($p < 0.025$). Mice receiving 4 µg $HgCl_2$ also had an increased percentage of Mac-1 (CD11b) positive cells ($p < 0.02$). These increases in memory/effector and Mac-1 positive cells were less pronounced than that typically observed in male BXSB mice, and these specific manifestations may depend largely on the presence of the *Yaa* gene. Mice receiving 0.4 µg $HgCl_2$ had no statistically significant increases in PBMC phenotypic markers (Table 5).

We also examined urinary protein after 32 weeks of treatment. Mice in the 4 µg and 0.4 µg groups had increased proteinuria compared with PBS controls ($p < 0.05$), whereas mice receiving 0.04 µg $HgCl_2$ had similar levels (Table 5). After 38 weeks of treatment, the surviving mice were sacrificed and kidney pathology and mercury levels were examined. Mice in the 4 µg group had the most severe glomerular changes (average score ± SE, 2.2 ± 0.6); this was the only group that differed significantly from the PBS controls (score 0.6 ± 0.1, $p = 0.03$; Figure 5). The two mice from the 40 ug group that were sacrificed at 20 and 23 weeks had only mild changes (score 1.3 ± 0.3), suggesting that the early mortality from mercury toxicity was probably unrelated to glomerulonephritis. Interestingly, this was the only group that developed vascular deposits of IgG, a finding characteristically observed with HgIA. Mice in the 0.4 and 0.04 µg groups had glomerular changes similar to those in the PBS group (scores of 0.8 and 0.6, respectively). Mercury levels in the kidney (Table 6), which reflects the overall exposure of the animal, were significantly elevated for each of the doses compared to the PBS controls ($p < 0.0001$). There was a

Table 5. Disease features in female BXSB after long-term exposure to $HgCl_2$.[a]

				Peripheral blood lymphocyte flow cytometry[b]				
No.	Treatment (µg $HgCl_2$)	Antichromatin Ab	Proteinuria mg/dL	$CD4^+$	$B220^+$	$CD4^+$ $CD44^{hi}$	$CD4^+$ $CD62L^{lo}$	$CD3^-$ $I\text{-}A^-$ $CD11b^+$
4	PBS	3.74 ± 2.02	32.2 ± 15.2	12.3 ± 1.5	72.1 ± 6.3	15.6 ± 3.6	15.9 ± 5.1	0.7 ± 0.5
4	0.04	4.81 ± 3.26	28.4 ± 9.2	12.7 ± 0.8	73.4 ± 6.9	17.5 ± 5.1	15.2 ± 1.9	0.8 ± 0.3
4	0.40	10.70 ± 4.10‡	78.4 ± 47.0*	13.4 ± 3.6	74.2 ± 6.4	14.9 ± 3.9	15.8 ± 6.0	1.2 ± 0.5
3	4.00	12.36 ± 4.80‡	85.3 ± 24.0*	11.6 ± 1.3	74.1 ± 2.9	29.7 ± 3.1#	22.0 ± 3.6	2.3 ± 0.6§

p-Values are from comparison of mercury-treated groups with PBS-treated group.
[a]Mice (8 weeks of age) were injected sc twice per week until 40 weeks of age; values given as mean ± 1SD. [b]Percent of live gated cells. *$p < 0.05$. §$p < 0.02$. ‡$p < 0.025$. #$p < 0.005$.

strong positive correlation between $HgCl_2$ dosage and kidney mercury levels ($R = 0.99$).

Discussion

In the present study, exposure of BXSB mice to mercury was shown to induce accelerated systemic autoimmunity in both the highly susceptible male and less susceptible female. Although we (*19*) and others (*35*) have shown that $HgCl_2$ exposure can induce systemic autoimmunity in other lupus-prone mice, in each instance the strains tested were susceptible to HgIA by virtue of their H-2 haplotypes alone. The current findings are significant in that the $H\text{-}2^b$ haplotype of the BXSB does not predispose to HgIA (*17,18*). Therefore, induction of systemic autoimmunity by $HgCl_2$ exposure clearly implicates other susceptibility genes, most likely those related to spontaneous lupus.

This study also demonstrates that exposure of BXSB mice to either a short course of high-dose mercury or lower doses over a long period could trigger systemic autoimmunity. For female BXSB mice, both the age of initial exposure and the dose of mercury were shown to influence the degree of disease exacerbation, particularly for antichromatin antibodies, an autoantibody closely associated with murine and human lupus (*31,36*). Moreover, a transient one-month exposure to mercury could elicit autoimmunity that persisted for many weeks after cessation of the xenobiotic and that had several features consistent with idiopathic disease.

Genetic studies show that both MHC and non-MHC genes can contribute to the expression of idiopathic systemic autoimmunity (*14*). In a previous study (*19*), comparison of the responses of MHC identical MRL-+/+ and AKR mice to mercury suggested that both MHC and non-MHC genes may contribute significantly to mercury toxicity. However, mercury exposure did not exacerbate humoral autoimmunity in the MRL-*lpr* mice, suggesting that $HgCl_2$ has little effect on mice that are already highly susceptible, or that it acts through the same anti-apoptotic mechanism as the Fas *lpr* mutation (*19*). In contrast, the present study found that the highly susceptible *Yaa*-expressing male BXSB mice were more sensitive to the autoimmune-promoting effects of $HgCl_2$ than female BXSB mice, which indicates that the addition of a strong autoimmune accelerating gene per se does not preclude xenobiotic acceleration of autoimmunity. Taken together with the MRL-Fas *lpr* results, this favors the possibility that $HgCl_2$ may promote HgIA by inhibiting the Fas-induced cell death of autoreactive cells. This is supported by recent evidence that $HgCl_2$ can protect against Fas-mediated cell death (*37*). Because mercury's effects are thought to be due primarily to reactivity to sulfhydryl groups, for which it has the highest binding (*38,39*), it is possible that mercury could be inhibiting cysteine proteases involved in the downstream events of Fas (*40,41*). Indeed, mutations of caspase 10 is one cause of the autoimmune lymphoproliferation syndrome in humans, a disease resulting from defective Fas-mediated apoptosis (*42*).

The accelerated autoimmunity in BXSB mice within the context of an MHC resistant to HgIA (*43*) suggests that autoimmunity in $HgCl_2$-exposed lupus-prone mice is due to acceleration of idiopathic disease and not elicitation of HgIA. In addition, the predominance of IgG2a and IgG2b subclasses in the antichromatin Ab response supports stimulation of a Th1-like cytokine response, which is more consistent with spontaneous disease in the BXSB (*32*). Although gene knockout studies have revealed that HgIA is dependent upon interferon-γ (*27*), disease expression in nonautoimmune-prone wild-type mice is associated with a Th2 response with elevations in IL-4 and autoantibodies of IgG1 subclass (*27,30*). The lack of IgG1 antichromatin antibodies in $HgCl_2$-exposed BXSB mice suggests that mercury is not eliciting a polyclonal B-cell response, nor is it promoting a Th2 response. The acceleration of autoimmunity by mercury is metal-specific: Another immune modulatory metal, nickel, did not accelerate autoimmunity in NZBWF1 or MRL mice (*19*). Similar experiments comparing the effects of mercury and nickel on BXSB mice also revealed that nickel was ineffective in accelerating autoimmunity in BXSB mice (data not shown). These observations suggest that $HgCl_2$ is acting as a trigger to complement genetic susceptibility in autoimmune-prone mice rather than simply inducing HgIA.

Maturation of splenic $CD4^+$ T cells from naive to activated phenotype, including increased expression of CD44 and loss of CD62L (*44*), has been associated with accelerated disease in male BXSB mice (*40,44*). Increases in such activated $CD4^+$ T cells were also observed in $HgCl_2$-exposed female BXSB mice. This appeared to be dose-dependent because it was found only in mice given 4 µg $HgCl_2$. Male BXSB also develop a strain-specific peripheral blood monocytosis (*45*). The cells express Mac-1 (CD11b) but not I-A, CD3, or B220 (*45*), and are thought to contribute to the pathogenesis of lupus in these mice (*46*). In contrast, increased percentages of $Mac\text{-}1^+$ cells are not found in young female BXSB mice (*45,46*). $HgCl_2$ exposure increased the percentage of $Mac\text{-}1^+$ cells in female BXSB mice, although the levels were low and reached statistical significance only for the group given 4 µg $HgCl_2$. Whether this small increase in the percentage of monocytes in the peripheral blood contributes to autoimmunity is questionable, especially because mice given 0.4 µg $HgCl_2$ had antichromatin antibodies in the peripheral circulation in the absence of any significant changes in PBMCs.

The relevance of these observations to human lupus will require further investigation because considerable debate exists regarding the sources and levels of mercury exposure within the human population (*16*). Consequently, few studies have attempted to equate environmentally relevant mercury exposure with systemic autoimmune disease

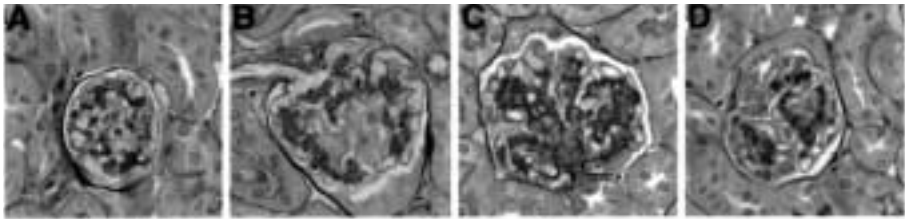

Figure 5. Representative PAS-stained sections of kidneys from female BXSB mice following long-term exposure to low doses of mercury. (*A*) PBS alone. (*B*) 40 µg $HgCl_2$ group. (*C*) 4 µg $HgCl_2$ group. (*D*) 0.4 µg $HgCl_2$ alone. Kidney sections were obtained from mice exposed to $HgCl_2$ for a total of 38 weeks, starting at 8 weeks of age. Glomeruli in (*C*) show substantial mesangial matrix expansion, thickening of glomerular capillary tufts, and slight increase in cellularity. Glomeruli in (*B*) show only minimal changes in the mesangial region. Magnification x400.

Table 6. Kidney mercury levels in female BXSB mice continuously exposed to $HgCl_2$.[a]

Treatment (µg $HgCl_2$)	No.	Kidney mercury (ng Hg/g wet weight) Mean ± 1SD	Range
PBS	4	12.4 ± 2.5	(10.5–16.0)
0.04	4	76.2 ± 6.0[##]	(71.6–84.2)
0.40	4	662.7 ± 84.7[##]	(569–734)
4.00	3	3643.7 ± 241.0[##]	(3,404–3,886)

p-Values are from comparison of mercury-treated groups with PBS-treated group.
[a]Mice (8 weeks of age) were injected sc twice per week until 46 weeks of age, when they were sacrificed to obtain kidneys for mercury determination. [##]$p < 0.0001$.

(*47*). Most studies have used levels of mercury to elicit autoimmunity in healthy (*8*) and autoimmune-prone mice (*19*) that, although possibly relevant to human occupational exposure (*16*), are greater than environmental exposure.

In attempting to determine the most relevant dose of mercury that would be comparable to environmental exposure, we focused on mercury levels in the kidney. There are several reasons for this: The kidney is a major site of accumulation of mercury in humans and mice (*16*,*48*). Reliable data exist on human kidney mercury levels with differing environmental exposures (*33*,*34*). Steady-state levels for mercury in mice are achieved after less than 4 weeks' exposure (*49*). In addition, the kidney is a major target organ in human and murine lupus and may constitute an initial site of tissue damage that leads to systemic exacerbation of disease (*50*). Levels of mercury in the kidneys in nonoccupationally exposed humans cover a broad range, from undetectable to 2,100 ng Hg/g wet weight of tissue (*33*,*34*), with the highest levels being found in dental amalgam bearers [average 433 ng Hg/g wet weight (*34*)]. The mercury levels in the kidneys of mice exposed to 0.4 μg $HgCl_2$/injection fall within the range found in nonoccupationally exposed humans. These mice had accelerated antichromatin antibodies and proteinuria, which suggests that environmentally relevant tissue levels of mercury could be associated with exacerbations of autoimmunity in genetically susceptible hosts.

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