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Effects of $HgCl_2$ on the Expression of Autoimmune Responses and Disease in Diabetes-Prone (DP) BB Rats

LINDA L. KOSUDA[a], DALE L. GREINER[a,b] and PIERLUIGI E. BIGAZZI[a,*]

[a]*Department of Pathology, University of Connecticut Health Center, Farmington, CT 06030;* [b]*Department of Medicine, University of Massachusetts, Worcester, MA 01655*

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Repeated exposure of Brown Norway (BN) rats to relatively low doses of $HgCl_2$ induces autoantibodies to renal antigens (e.g., laminin) and a membranous glomerulonephropathy characterized by proteinuria. In contrast, Lewis (LEW) rats are "resistant" to the autoimmune effects of mercury and, when exposed to this metal, are protected against experimental autoimmune encephalomyelitis (EAE) and Heymann's nephritis. To date, there is no information on "suppressive" effects of mercury in naturally occurring (so-called "spontaneous") rat models of autoimmune disease. Therefore, we have administered $HgCl_2$ to diabetes-prone (DP) BB rats, animals that spontaneously develop both insulin-dependent diabetes mellitus (IDDM) and thyroiditis. We found that DP rats treated with mercury or water for a period of 40-125 days developed autoantibodies to thyroglobulin, with a higher incidence in $HgCl_2$-injected animals (92% vs. 56% in H_2O-injected controls). A novel finding of our study was the detection of autoantibodies to laminin in the same rats, again with an increased incidence after $HgCl_2$ treatment (83% vs. 44%). IgG2a was the most frequently detected isotype of antibodies to laminin, followed by IgG1, IgG2b and IgG2c. The IgG isotype profile suggests that treatment with $HgCl_2$ may activate both Th1 and Th2 lymphocytes in BB rats. In spite of these stimulatory effects on autoantibody responses, we found that there was no difference in the incidence of IDDM and thyroiditis between $HgCl_2$-treated and control animals.

We conclude that the suppressive effects of mercury previously observed in EAE and Heymann's nephritis of LEW rats do not occur in "spontaneous" autoimmune IDDM and thyroiditis of BB rats. Therefore, immune suppression caused by $HgCl_2$ cannot be considered a common phenomenon, but may be a genetically determined characteristic of LEW rats, possibly related to a specific or unique cytokine profile of this particular rat strain. In contrast, while mercury does not seem to recruit, induce or rescue regulatory T cell function in DP rats, it does stimulate autoantibody responses in these animals.

Keywords: Diabetes mellitus, thyroiditis, autoantibodies to thyroglobulin, autoantibodies to laminin, IgG isotypes, IgG1, IgG2a, IgG2b, IgG2c, xenobiotics, Th1 lymphocytes, Th2 lymphocytes

*Corresponding Author: Pierluigi E. Bigazzi, M. D., Department of Pathology, University of Connecticut Health Center, Farmington, CT 06030 (USA).

INTRODUCTION

Experimental administration of mercury to rodents provides excellent animal models of autoimmunity induced by xenobiotics.[1] Rats of the Brown Norway (BN), MAXX and DZB strains, exposed to relatively low doses of $HgCl_2$, develop a membranous glomerulonephropathy, characterized by proteinuria and the production of autoantibodies to epitopes of the renal glomerular basement membrane (GBM), including laminin.[2–5] On the other hand, Lewis (LEW) rats are "resistant" to these autoimmune effects of mercury.[6] In addition, $HgCl_2$ induces a nonspecific immunosuppression in LEW rats, resulting in their protection from Heymann's nephritis and experimental autoimmune encephalomyelitis (EAE), two animal models of autoimmunity obtained by immunization with organ-specific antigens.[7–9] These findings have suggested that xenobiotics mimicking the effects of mercury could be utilized as therapeutic immunosuppressive agents.[10,11]

However, the ability of $HgCl_2$ to regulate autoimmune disease that occurs naturally, i.e., without the need for immunization with autoantigens or other experimental manipulations, is still uncertain. Inbred strains of rats provide only a few examples of "spontaneous" genetically determined organ- or tissue-specific autoimmunity.[12] Currently, the available rat models are insulin-dependent diabetes mellitus (IDDM), observed in BB rats, and autoimmune thyroiditis, detected in both BB and BUF rats. Since BB rats are naturally affected by two autoimmune diseases, we have investigated the effects of $HgCl_2$ in these animals. The frequency of spontaneous IDDM in "diabetes-prone" (DP) rats is ≈80%, with the majority of cases occurring between 60 and 120 days of age.[13,14] Autoimmune thyroiditis develops independently of IDDM and is observed in 5–100% of DP rats.[15] The expression of autoimmunity in BB rats appears to be determined by the relative balance of autoreactive effector T cells and regulatory (possibly RT6+) T cells.[14] DP rats with IDDM may be characterized by a predominance of Th1-type lymphocytes, since recent studies of cytokine message expression in islets and thyroids of DP rats have detected the presence of IFN-γ and IL-12 mRNA, suggesting an inflammatory response mediated by Type 1 cytokines.[16] In the present study, we have examined whether $HgCl_2$ affects the expression of autoimmunity in these animals. In contrast to the observations in the LEW rat models of EAE and Heymann's nephritis, $HgCl_2$ does not delay or alter the onset or frequency of IDDM and thyroiditis in DP BB rats. On the other hand, long-term exposure of these rats to mercury stimulates autoantibody responses to thyroglobulin and laminin.

MATERIALS AND METHODS

Experimental Animals

Female DP rats were obtained from the BB rat colony of the University of Massachusetts Medical Center, Worcester, MA. These animals were divided in three groups, as follows:

The *first* group (BB#1) comprised 10 DP rats housed under "conventional" conditions. They were 74–87 (mean 78 ± 4) days of age at the beginning of the experiment and were sacrificed at 91–104 (mean 95 ± 4) days of age. These animals were serologically identical to "viral antibody free" BB rats (see below), except that some tested serologically positive for Kilham rat virus, an environmental pathogen that influences frequency and kinetics of IDDM in BB rats.[17,18]

The *second* group (BB#2) comprised 30 DP rats, that were totally "viral antibody free" (VAF), i.e., certified to be free of Kilham rat virus, Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, H1 (Toolan's virus), GD7, Reo-3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus and *Encephalitozoon cuniculi*. These rats were 32–34 (mean 33.5 ± 0.9) day-old at the beginning of the experiment and were sacrificed at 69–124 (mean 85.7 ± 18) days of age.

The *third* group (BB#3) comprised 21 DP rats, also totally "VAF". They were 33–35 (mean 34 ± 1)

day-old at the beginning of the experiment and were sacrificed at the onset of diabetes, that was detected at 73–139 days of age. Only 5 rats of this group did not develop clinical signs of IDDM during the experiment and were sacrificed at 158–160 days of age (see below under Administration of mercury).

All rats were housed in plastic cages with wood shavings, in an automated light cycle environment (12:12 hr) and received standard rat diet and water *ad libitum*. They were maintained in accordance with the regulations of the University of Massachusetts and the University of Connecticut Health Center and the recommendations of the *Guide for the care and use of laboratory animals* (Department of HEW Publication NIH 78–23, 1985).

Administration of Mercury

Rats were anesthesized with ether or methoxyflurane (metofane) inhalation anesthesia prior to treatment. They were injected subcutaneously (s.c.) on the abdomen with 0.1 ml of a mercuric chloride solution ($HgCl_2$, 1 mg/ml) per 100 g bw, as previously described.[19] Rats injected with $HgCl_2$ are defined in the text as "M" rats. Control rats, of same age and weight as those injected with $HgCl_2$, were injected s.c. with distilled H_2O at pH 4.2 following the same schedule. These animals are referred to as "W" rats.

The effects of a relatively brief exposure to mercury in "conventional" DP rats were studied on 5 DP rats of the *first* group (BB#1), that were injected 6 times with mercury (100 μg $HgCl_2$/100 g bw/rat per injection for a total dose of 600 μg $HgCl_2$/100 g bw/rat). As controls, 5 DP rats of the same group received 6 injections of distilled H_2O following the same schedule. To determine the effects of a similar exposure to mercury on "VAF" animals of younger age, 16 DP "VAF" rats of the *second* group (BB#2) received 6 injections of mercury (100 μg $HgCl_2$/100 g bw/rat per injection). Controls were 14 DP rats injected with water. Finally, 12 DP "VAF" rats of the *third* group (BB#3) were used to study the immunotoxic effects of a protracted mercury exposure in "VAF" DP BB animals. In this group, 10 rats were injected with mercury until the onset of diabetes (as measured by glycosuria and hyperglycemia) and received 16–43 injections (100 μg $HgCl_2$/100 g bw/rat per injection, for a total dose of 1600–4300 μg $HgCl_2$/100 g bw/rat). As controls, 6 DP "VAF" rats were injected with water. All these animals were sacrificed at 73–139 days of age. Only 5 rats of Group BB#3 (2 mercury-treated and 3 water-treated) did not develop clinical signs of IDDM and therefore treatment was continued for a total of 51 injections of $HgCl_2$ or H_2O, until the rats were sacrificed at 158–160 days of age.

Clinical Evaluation of IDDM

As previously described,[16] rats were screened for glycosuria (Tes-Tape, Eli Lilly, Indianapolis, IND) and diabetes was diagnosed on the basis of a plasma glucose concentration >11.1 mM.

Histopathology

Sections of thyroid, pancreas and adrenal, stained with H&E, were examined by light microscopy for inflammatory infiltrates. Thyroiditis was evaluated by a modification of a previously described procedure.[20] Sections were graded from 0 to 4+, depending on the extent of lymphocytic infiltration and tissue lesions. For example, normal thyroids were scored as 0. Thyroids exhibiting definite thyroiditis, but with less than 30% of the parenchyma replaced by mononuclear cells, were scored as 1+. Thyroids exhibiting 30–50% loss of normal structure were scored as 2+. Thyroids with 50–70% inflammatory infiltration were scored as 3+ and those with more than 70% as 4+. Similar criteria were used for the adrenals. Sections of pancreas were examined for the presence of insulitis or "end-stage" islets, as previously described.[21]

Detection of Autoimmune Responses

Autoimmune responses to antigens of thyroid and kidneys were evaluated by ELISA for antibodies

to thyroglobulin or laminin and direct immunofluorescence for renal immunoglobulin deposits, as follows:

ELISA for autoantibodies to thyroglobulin

Circulating autoantibodies to rat thyroglobulin were detected by ELISA.[22] In brief, sera from "M" and "W" rats were incubated in ELISA plates that had been previously coated with rat thyroglobulin (0.1 μg/100 μl/well). Rat thyroglobulin was purified as previously described.[20] All sera were tested in duplicate wells using 1:100 dilutions. After 30 minutes of incubation and appropriate washing, enzyme-labeled antibodies (horseradish peroxidase-conjugated affinity-purified $F(ab')_2$ fragment of goat antibodies to rat IgG, H&L chains, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used to detect the binding of rat anti-thyroglobulin. ABTS substrate was added and plates were read using a MR600 Dynatek ELISA Reader (Dynatech Instruments Inc., Torrance, CA) at OD_{410} after 5 and 10 minutes of incubation. The "positive/negative cut-off" line was determined by established procedures.[23] Positive control sera for autoantibodies to thyroglobulin were obtained from BUF rats with autoimmune thyroiditis.[20] Negative reference sera were obtained from BB rats before the start of mercury or water treatment. They were used to calculate the mean negative value. Rat sera were considered to contain autoantibodies to thyroglobulin when they showed a definite OD_{410} increase, that was above the mean negative value plus two standard deviations of the mean.

ELISA for antibodies to laminin and their IgG isotypes

Circulating antibodies to mouse laminin were detected by ELISA.[24] In brief, sera from "M" and "W" rats were incubated in ELISA plates that had been previously coated with laminin (1 μg/100 μl/well). Mouse laminin was obtained courtesy of Dr. Hinda Kleinman (NIH, NIDR).[25,26] All sera were tested in duplicate wells using 1:100 dilutions. The binding of rat anti-laminin was detected as described above for autoantibodies to thyroglobulin, with the difference that reading of plates was performed after 10, 15 and 20 minutes of incubation with the substrate. Positive control sera for antibodies to laminin were obtained from mercury-treated BN rats.[24] Negative reference sera were obtained from BB rats before the start of mercury treatment and from "W" rats. Criteria to define positive and negative sera were similar to those described above for autoantibodies to thyroglobulin. Circulating autoantibodies to rat laminin were also detected by ELISA following a procedure similar to the one described above, with the exception that ELISA plates were coated with rat laminin (1 μg/100 μl/well, Gibco BRL, Gaithersburg, MD). In experiments to compare reactions against rat and mouse laminin, three dilutions (1:100, 1:1,000 and 1:5,000) of positive and negative sera were tested in ELISA plates that had some wells coated with rat laminin (1 μg/100 μl/well) and other wells with mouse laminin (1 μg/100 μl/well). Detection of binding was performed as usual, but readings were obtained at OD_{405} after 5, 10, 15 and 20 minutes of incubation with the substrate in a constant temperature microplate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA).

Isotypes of IgG antibodies to mouse laminin were detected by a modification of the ELISA described above, based on the use of biotinylated mouse monoclonal antibodies (mAbs) and horseradish peroxidase-labeled streptavidin (HRP-SA). For the detection of IgG1 and IgG2a antibodies to laminin, sera were diluted 1:100 and incubated for 30 minutes, followed by washing and a 30 minute incubation with biotinylated mAbs to rat IgG1 or IgG2a (Caltag Laboratories, South San Francisco, CA) diluted 1:100. Since IgG2b and IgG2c antibodies were more difficult to detect, we incubated for 30 min both 1:10 and 1:100 dilution of sera, followed by washing and a 30 min incubation with biotinylated mAbs to rat IgG2b or IgG2c (Caltag, Laboratories, South San Francisco, CA) diluted 1:100. Bound biotinylated mAbs were then detected by HRP-SA (Caltag Laboratories, South San Francisco,

CA) diluted 1:1,000. Each test included various controls, e.g., "irrelevant" rat sera and mouse mAbs, as well as biotinylated mAb and HRP-SA alone. Plates were read and results evaluated as described above.

Immunohistopathology of rat kidneys

Demonstration of rat IgG deposits in renal glomerular basement membrane (GBM), tubular basement membrane (TBM) and/or mesangium was performed by direct immunofluorescence (DIF) as previously described.[24] In brief, cryostat sections of rat kidney were stained with FITC-conjugated rabbit antibodies to rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and read using a Leitz Dialux fluorescence microscope equipped with epi-illumination. Duplicate sections from each rat were examined and defined as positive when staining of IgG was present in the GBM or other renal structures. The intensity of DIF staining in each kidney section was subjectively graded on a scale from 0 = negative to 4 = highly positive. Sections with a grading of 1 or higher were considered positive.

Flow Cytometry (FCM) of Rat Lymphocytes

Spleen, lymph node and thymus cell suspensions were obtained and stained for FCM as previously described.[27] Cell suspensions were labeled with anti-RT6.1 or anti-RT6.2 rat mAb and developed for immunofluorescence with an $F(ab')_2$ fragment of a FITC-conjugated goat anti-rat IgG (heavy and light chain specific) (Caltag Laboratories, San Francisco, CA). Controls included the FITC conjugate alone and "irrelevant" primary rat mAb developed with the same secondary reagent: values obtained from these controls represent surface Ig positive (B) lymphocytes and were subtracted from the counts of RT6+ cells. Cells were also stained with mAb to the rat lymphocyte antigens OX19 (anti-CD5, pan-T cells), OX8 (anti-CD8, T cell subset and natural killer cells), W3/25 (anti-CD4 T cell subset and monocytes), OX39 (anti-IL-2 receptor) and OX4 (anti-common class II MHC). When cells were labeled with these mouse mAb, they were developed with a $F(ab')_2$ fragment of a FITC-conjugated goat anti-mouse IgG antibody that had been absorbed by affinity chromatography on a rat IgG column to remove cross-reacting antibodies. Controls routinely included the FITC conjugate alone and irrelevant mouse mAb. Cell suspensions were fixed before FCM using 1% paraformaldehyde and then analyzed using a FACScan (Becton Dickinson, Mountain View, CA), according to relative low angle light scatter and relative fluorescence intensity as described previously.[27] Dead cells and contaminating red blood cells were excluded from analysis by electronic gating. At least 50,000 nucleated cells were analyzed for relative fluorescence intensity.

Statistical Evaluation of Data

All ELISA and DIF tests were performed in duplicates and the data obtained from all experimental (or control) groups expressed as the mean ± standard error of the mean (s.e.m.). FCM analysis was performed on single samples from each rat and the data from all groups presented as the mean ± s.e.m.. Histopathology, immunohistopathology, ELISA and FCM results were statistically evaluated by one-way analysis of variance (ANOVA), followed by post-hoc means tests (Fisher's Protected Least Significance Difference, Scheffe's S and Bonferroni's multiple comparison procedures). Contingency Tables and Chi-square test were used to compare percentages of rats with circulating autoantibodies after treatment with mercury or water. Correlation analysis was performed to identify possible relationships between the various data (FCM, serum autoantibodies, and histopathology). Two computer programs (StatView 4.1 and SuperANOVA, Abacus Concepts, Inc., Berkeley, CA) have been used for statistical analysis of data.

RESULTS

Circulating Autoantibodies to Thyroglobulin

We first examined all sera by ELISA for autoantibodies to thyroglobulin, since they have been previously observed in DP rats.[28–30] Figure 1 shows

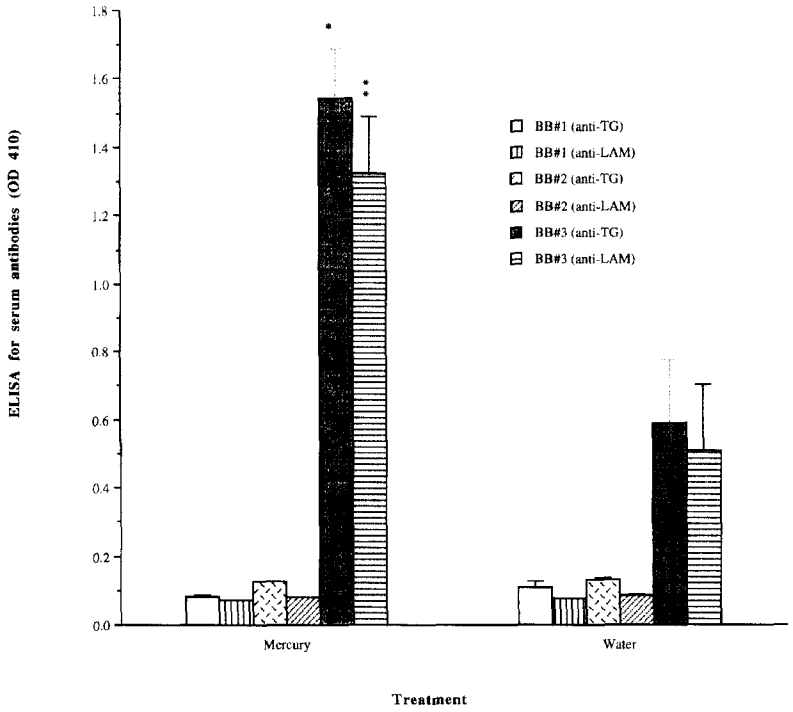

FIGURE 1 Circulating antibodies to rat thyroglobulin (anti-TG) and mouse laminin (anti-LAM) in DP BB rats treated with mercury or water. The mean OD_{410} (±SEM) obtained with sera from all water-treated DP rats of Group BB#3 was higher than that obtained from similarly treated controls of Groups BB#1 and BB#2. The mean OD_{410} (±SEM) obtained with sera from all mercury-treated DP rats of Group BB#3 was higher than that observed in similarly treated rats of Groups BB#1 and BB#2. In addition, mercury-treated DP rats of Group BB#3 gave a higher mean OD_{410} than water-treated controls of the same group (*$p = 0.0002$; **$p = 0.0029$).

that the mean OD_{410} obtained from all sera of Groups BB#1 and BB#2 was quite low, without a significant difference between mercury- and water-treated rats ($p = 0.5025$ for Group BB#1 and $p = 0.5370$ for Group BB#2). Pre-treatment sera as well as sera from untreated rats had a similar mean OD_{410} (data not shown), therefore, we concluded that DP rats of groups BB#1 and BB#2 did not produce autoantibodies to thyroglobulin. On the other hand, sera obtained at sacrifice of Group BB#3 rats (that were followed for a longer time to assess the effects of a protracted exposure to mercury) had a much higher mean OD_{410}. As shown in Figure 1, mercury-treated DP rats of this group gave a mean $OD_{410} = 1.545 \pm 0.144$ and water-treated rats a mean $OD_{410} = 0.591 \pm 0.184$, with a significant ($p = 0.0002$) difference between the two treatments. Control sera obtained from BUF rats with autoimmune thyroiditis and previously shown to contain autoantibodies to thyroglobulin gave a mean OD_{410} of 1.630 ± 0.15 (data not shown).

Individual rat sera were examined by comparison with reference sera obtained from the same animals before the start of treatment and from "normal" untreated rats. They were considered to contain autoantibodies to thyroglobulin when there was a definite OD_{410} increase after treatment (above the mean reference value plus two standard deviations of the mean). Table I shows that autoantibodies to rat thyroglobulin developed in 92% of "M" and 56% "W" DP rats of Group BB#3 (Chi Square p value = 0.0545).

Circulating Antibodies to Laminin and their IgG Isotypes

Earlier investigations have shown that, in addition to thyroglobulin antibodies, BB rats produce

TABLE I Circulating autoantibodies in diabetes-prone (DP) rats of Group BB#3

Number of rats[a]	*Treatment*[b]	*Number of rats with antibodies to*		
		Rat thyroglobulin[c]	*Mouse laminin*[c]	*Rat laminin*[c]
12	$HgCl_2$	11 (1.671 ± 0.126)	10 (1.574 ± 0.146)	9 (0.756 ± 0.096)
9	H_2O	5 (1.051 ± 0.300)	4 (1.073 ± 0.350)	2 (0.706 ± 0.041)

[a]A total of 21 "viral antibody free" female DP rats, aged 33-35 days at beginning of experiment and sacrificed at 73-160 days of age.
[b]Rats were treated for 40-125 days with 16-51 injections of $HgCl_2$ or H_2O.
[c]In brackets the mean OD_{410} ± s.e.m. obtained in rats with circulating antibodies.

autoantibodies to antigens of smooth muscle and gastric parietal cells, but not against adrenal and islet cell antigens.[28,29] On the other hand, mercury-treated Brown Norway (BN) rats are known to produce antibodies to laminin.[19] Therefore, we tested all DP rat sera by ELISA against mouse laminin and found that animals of Group BB#3 gave a higher mean OD_{410} as compared to the other groups. As shown in Figure 1, the mean OD_{410} of all sera from mercury-treated DP rats of this group was 1.322 ± 0.169 and from water-treated rats 0.508 ± 0.194, with a significant ($p = 0.0029$) difference between the two treatments. As a comparison, positive control sera obtained from BN rats with $HgCl_2$-induced autoimmunity and previously found to contain antibodies to laminin, gave a mean OD_{410} of 1.140 ± 0.23 (data not shown). Circulating antibodies to laminin were determined by comparison with pre-treatment samples and reference sera from "normal" untreated rats. There was a very high correlation (= 0.952) between the presence of circulating autoantibodies to thyroglobulin and antibodies to laminin. Table I shows that 83% of DP rats of Group BB#3 had serum antibodies to mouse laminin, whereas only 44% of H_2O-treated control animals of the same group were similarly positive at the time of sacrifice (Chi Square $p = 0.0614$). Figure 1 also demonstrates that DP rats of Groups BB#1 and BB#2 did not develop antibodies to laminin, irrespective of treatment.

Sera from rats of Group BB#3 were also tested for possible reactivity against rat laminin. We found that most sera with antibodies to mouse laminin also gave positive reactions with rat laminin, i.e., contained autoantibodies to laminin (83% of $HgCl_2$- and 22% of H_2O-treated DP rats). OD_{410} values obtained with those sera were consistently higher against mouse laminin (Table I). When repeated in the same assay using a microplate reader capable of maintaining a constant temperature, there was no significant difference in reactions with mouse or rat laminin at a dilution of 1:100 ($p = 0.3083$) (Figure 2). However, OD_{405} values obtained with sera diluted at 1:1,000 and 1:5,000 were higher against mouse laminin than against rat laminin ($p = 0.0273$ and $= 0.0514$, respectively).

We next determined the IgG subclass of antibodies to mouse laminin using isotype-specific monoclonal antibodies.[31,32] In general, rats with the highest levels of IgG antibodies to laminin (as estimated by their OD_{410}) were highly positive for the various isotypes. Most animals produced laminin antibodies of the IgG2a and IgG1 subclasses, but some also had detectable levels of IgG2b and IgG2c. As shown in Figure 3, 11 of 12 "M" and only 5 of 9 "W" rats produced IgG2a laminin antibodies (Chi Square $p = 0.0545$). Circulating IgG1 antibodies to laminin were detected in 8 "M" and 1 "W" DP rats (Chi Square $p = 0.0109$). Only 3 DP "M" rats had high levels of IgG2b antibodies when tested at the standard serum dilution of 1:100 (data not shown). However, 8 "M" rats (but none of the "W" controls) showed significant levels at 1:10 dilutions (Chi Square $p = 0.0019$). Finally, IgG2c antibodies were not detected in any sera tested at 1:100 dilutions. Only 3 "M" and 1 "W" DP rats had significant OD_{410} at 1:10 ($p < 0.0001$ and $= 0.0055$, respectively). Thus, DP rats produce autoantibodies to laminin that belong mostly to the IgG1 and IgG2a isotypes. In addition, $HgCl_2$ can stimulate autoantibody responses to laminin in DP rats, with

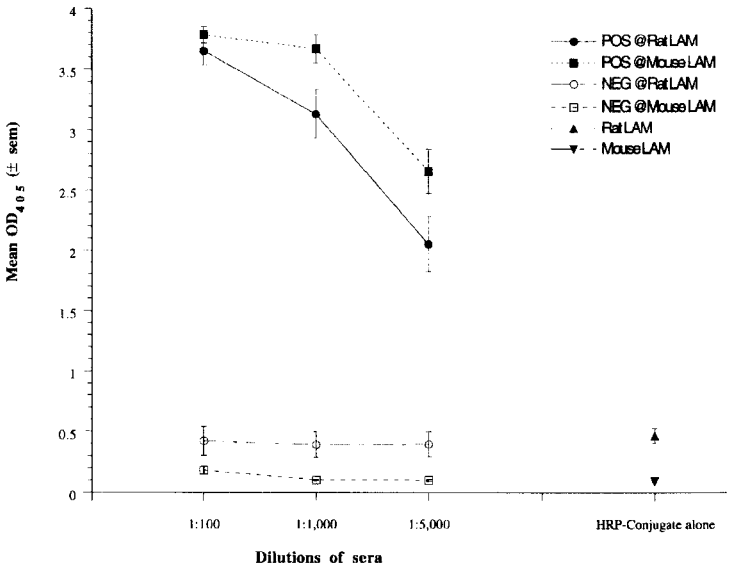

FIGURE 2 Comparison of reactions against mouse and rat laminin using three different dilutions of DP rat sera (Group BB#3). Results are expressed as mean OD_{405} ± SEM. After 15 minutes of incubation with substrate in a constant temperature microplate reader, there was no significant difference at sera dilutions of 1:100, but dilutions of 1:1,000 and 1:5,000 gave higher values with mouse than rat laminin. OD_{405} labeled POS @Rat LAM and POS @Mouse LAM were obtained with DP rat sera that had given positive reactions against these antigens. NEG @Rat LAM and NEG@Mouse LAM were obtained with negative sera. OD_{405} labeled Rat LAM and Mouse LAM were obtained after incubation of these antigens with HRP-conjugate alone (in the absence of rat sera).

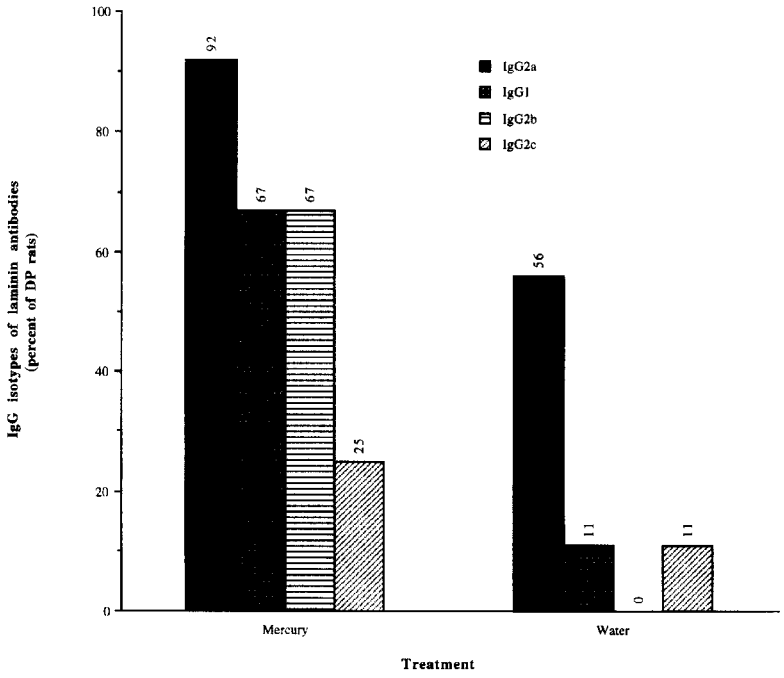

FIGURE 3 IgG isotypes of antibodies to mouse laminin in sera of DP rats from Group BB#3. Circulating IgG2a antibodies were detected in 56% of water-treated and 92% of mercurytreated rats. Similarly, there were significant increases in the percentages of mercury-treated DP rats with laminin antibodies of IgG1, IgG2b and IgG2c isotypes.

TABLE II Effects of $HgCl_2$ on autoimmune disease of DP BB rats

Group[a]	*Number of rats*	*Treatment*[b]	*Percentage of rats with*			
			IDDM	*Islet pathology*[c]	*Thyroiditis*	*Adrenalitis*
BB#1	5	M	60	80	40	0
BB#1	5	W	60	100	60	0
BB#2	16	M	88	94	56	ND[d]
BB#2	14	W	86	93	64	ND[d]
BB#3	12	M	83	100	17	ND[d]
BB#3	9	W	67	100	11	ND[d]

[a]BB#1: 10 non-"VAF" DP rats, aged 74-87 days at beginning of experiment and sacrificed at 91-104 days of age; BB#2: 30 "VAF" DP rats, aged 32-34 days at beginning of experiment and sacrificed at 69-124 days of age; BB#3: 21 "VAF" DP rats, aged 33-35 days at beginning of experiment and sacrificed at 73-160 days of age.
[b]"M" = $HgCl_2$ – treated, "W" = H_2O – treated.
[c]Insulitis and/or "end-stage" islets.
[d]ND: not done.

all IgG isotypes (including IgG2b and IgG2c) being produced.

Development of IDDM and Insulitis

As shown in Table II, 6/10 DP rats of Group BB#1 (that had been housed under "conventional" conditions and were seropositive for Kilham rat virus) had clinical manifestations of IDDM (glycosuria and hyperglycemia), irrespective of exposure to mercury. Inflammatory infiltration of the islets was observed in 4/5 $HgCl_2$-injected ("M") and 5/5 H_2O-injected ("W") rats. Severity of insulitis, subjectively evaluated by extent and intensity of inflammatory infiltrates, was not different between experimental and control rats. Similar results were observed in mercury- and water-treated VAF DP rats of Group BB#2 followed to 69–124 days of age (Table II).

Finally, IDDM developed in 10/12 "M" and in 6/9 "W" VAF DP rats of group BB#3 (Table II, Chi Square $p = 0.3749$). These results agree with the reported cumulative incidence of spontaneous IDDM in VAF DP rats, that is ≈80% by 120 days of age.[33] There was also no significant difference in the mean age of onset, that was 88.5 days (±4) in "M" and 83.3 days (±3) in "W" rats ($p = 0.339$). Diabetic rats had small pancreatic islets, that were reduced in number and showed "end-stage" islet morphology. All the non-diabetic rats sacrificed at 158–160 days of age had severe insulitis. Thus, exposure to mercury did not affect the time of onset or frequency of spontaneous IDDM in DP BB rats.

Development of Thyroiditis and Adrenalitis

Table II shows that mononuclear cell infiltration of the thyroid was present in all three groups of DP rats, with no differences in the frequency of inflammatory infiltrates between mercury- and water-treated animals. In addition, no significant differences in the extent and intensity of inflammatory infiltrate were noted (data not shown).

The adrenals of all DP rats of Group BB#1 were normal. Since we found no histopathologic alternations in the adrenals of Group BB#1, we did not perform additional studies of these organs.

Renal Immunopathology

Direct immunofluorescence of kidney sections did not reveal significant immune deposits in any rat. We paid particular attention to kidneys from rats with circulating autoantibodies to laminin, since BN rats with similar antibodies develop immune deposits (containing antibodies to laminin) at the level of the renal GBM and TBM.[3,5,34] In spite of repeated examinations, immunoglobulin deposits (either linear or granular) were not observed in kidneys of DP rats.

TABLE III Effects of $HgCl_2$ on cervical lymph node lymphocytes of DP BB rats

Group[a]	*Treatment*[b]	*Number of* cells ($x10^6$)	*%RT6.1*	*%B*	*%CD8*	*%CD4*	*%T*
BB#1	M	16 ± 3	0	59 ± 2	4.4 ± 0.5	33 ± 4	28 ± 2.7
BB#1	W	9 ± 2	1.2 ± 1.1	58 ± 3	3.7 ± 0.5	27 ± 3	26 ± 3.5
BB#3	M	13 ± 2	0.8 ± 0.9	66 ± 2	9.2 ± 2.3	20 ± 1	422 ± 1.8
BB#3	W	10 ± 2	2.4 ± 1.5	56 ± 4	8.9 ± 1.5	24 ± 1	26 ± 1.9

[a]see Table II.
[b]M = rats injected with $HgCl_2$; W = rats injected with H_2O.

FCM Analysis of Lymphocytes and Thymocytes

Table III shows that, as expected, untreated DP rats of Group BB#1 and BB#3 had low numbers of lymphocytes in peripheral lymphoid organs and experienced severe deficiencies in the number of RT6.1+ cells.[35] In addition, both mercury-treated and control DP rats had low percentages of CD8+ T lymphocytes. There was a slight decrease in the percentage of CD4+ T cells ($p = 0.0502$) and a slight percentage increase of B lymphocytes ($p = 0.0322$) in mercury-treated DP rats of group BB#3, but no significant numerical changes ($p = 0.6907$ and $= 0.2234$, respectively). Correlation analysis did not reveal high degrees of relationship between FCM data and circulating autoantibodies. Thus, in contrast to our observations in BN rats,[19,24,36] exposure to mercury did not cause striking and highly significant changes in numbers and percentages of peripheral lymphocyte subpopulations in DP BB rats.

We also evaluated thymocyte numbers, since BN rats treated with $HgCl_2$ have thymus atrophy as well as a decreased percentage of double positive (CD4+CD8+) thymocytes.[27] These changes are not found in LEW rats. Similarly, there were no significant differences in numbers of thymocytes between "M" and "W" DP rats of Group BB#1 (Figure 4). However, "M" DP rats of Group BB#3

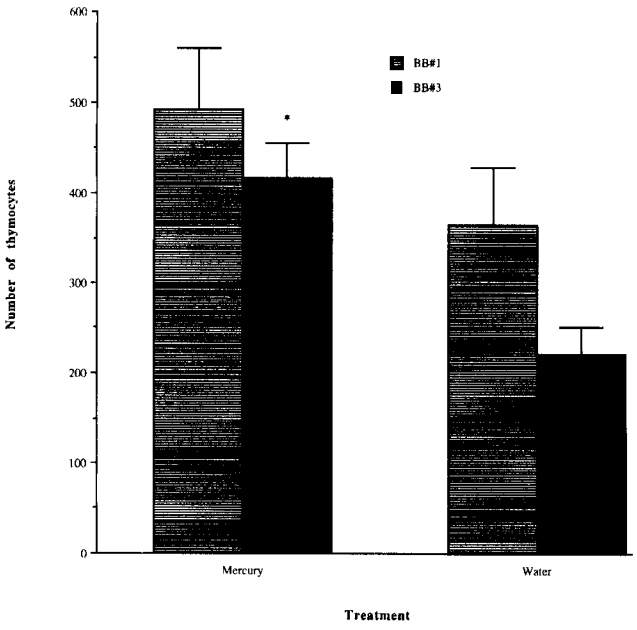

FIGURE 4 Number of thymus cells ($x10^6$, expressed as mean ± SEM) in DP rats from Groups BB#1 and BB#3. There was no difference in thymocyte numbers between mercury-treated and water-treated DP rats of Group BB#1. On the other hand, mercury-treated DP rats of Group BB#3 had significantly higher numbers of thymus cells than water-treated controls of the same group (*$p = 0.0013$).

had significantly higher numbers of thymocytes than "W" rats of the same group ($p = 0.0013$).

DISCUSSION

In the present study, we examined the effects of mercury treatment on the autoimmune syndromes of DP BB rats. Time of onset, frequency or severity of IDDM and thyroiditis did not change in the spontaneously hyperglycemic DP BB rat even after prolonged exposure to $HgCl_2$. These results are in marked contrast with the ability of mercury to induce lymphocyte alterations and autoimmunity in "mercury-susceptible" strains, such as the BN rat.[19,24,36] In addition, they differ with reports that mercury acts as an immunosuppressive compound for experimentally induced autoimmunity in "mercury-resistant" LEW strain rats.[7–10,37–39] It was these latter observations from autoimmune models experimentally induced in the LEW rat that stimulated our studies of possible immunoregulatory activity of mercury in other rat strains. To attempt to generalize these findings to "spontaneous" autoimmune disorders, we tested the effects of mercury in the autoimmune-prone strain of BB rats. Our results show that the administration of mercury had no inhibitory effects on the development and progression of insulitis and IDDM in DP BB rats. In addition, mercury treatment did not induce autoimmunity to adrenals in these animals.

A novel finding of our study is the detection of autoantibodies to laminin in DP BB rats, with an increased incidence after $HgCl_2$ treatment. Circulating antibodies to mouse laminin were found in the majority of mercury-treated animals and in a smaller percentage of controls of Group BB#3. Sera from the same animals also reacted against rat laminin, i.e., contained autoantibodies to this antigen. There was no significant correlation between the presence of autoantibodies to laminin and IDDM. In addition, DP rats with high levels of circulating IgG autoantibodies to laminin did not show any inflammatory kidney disease. Laminin, an evolutionary conserved protein, is structurally very similar in mouse and rat.[40,41] To date, antibodies to laminin have only been reported to be present in the serum of mercury-treated BN, MAXX and DZB rats as well as Sprague Dawley (SD) rats exposed to cadmium, but not in untreated animals.[42,43] Interestingly, autoantibodies to laminin did not bind to either the renal GBM or TBM of DP rats, in contrast to the findings in mercury-treated BN, MAXX and DZB rats. However, a similar lack of renal immune deposits has been observed in cadmium-treated SD rats.[42] It is possible that the autoantibodies to laminin produced by DP and SD rats are directed against laminin epitopes that are not accessible for *in vivo* binding.

In addition to autoantibodies to laminin, DP rats of Group BB#3 had circulating autoantibodies to thyroglobulin. High levels of these autoantibodies were detected in animals that had no inflammatory thyroid disease, a lack of correlation that has been previously observed in both DP and BUF rats.[20,30] Our findings of autoantibodies to thyroglobulin and laminin, as well as older reports of autoantibodies to antigens of smooth muscle cells and gastric parietal cells,[28,29] suggest that BB rats are predisposed to the production of antibodies to a variety of autoantigens. Exposure to $HgCl_2$ did not inhibit and actually appears to have stimulated autoantibody responses of DP rats. A similar phenomenon has been recently observed in young (NZB × NZW)F1 hybrids, that showed mercury-induced polyclonal B-cell activation with increased autoantibody production and renal immune complex deposition.[44]

Antibodies to laminin were most commonly of the IgG1 and IgG2a subclasses. However, a considerable number of sera also contained antibodies of the IgG2b and IgG2c isotypes. To date, there is little information on the regulation of IgG isotype expression in rats. However, rat IgG2a may be induced by IL-4 similarly to what occurs for mouse IgG1.[45] Rat IgG2b and IgG2c are stimulated by IL-12, an effect largely dependent on IFN-γ production.[46] It has also been hypothesized that mercury stimulates Th2 cells in BN rats and Th1 cells in LEW rats.[47–50] Our findings suggest that in untreated DP rats with IDDM the production

of antibodies to laminin (mostly of the IgG2a isotype, with a small percentage of IgG1 and IgG2c) may be preferentially under the control of cytokines from Th2 lymphocytes. In addition, the IgG isotype profile suggests that treatment of DP rats with $HgCl_2$ primarily stimulates Th2 but may also activate Th1 cells, albeit to a lesser extent. Mercury-induced stimulation of Th2 lymphocytes did not affect the expression of IDDM, predominately a Type 1 cytokine-mediated disease.[16]

Finally, contrary to what happens in BN rats that show a lymphoproliferative response in their peripheral lymphocytes and a decrease in RT6+ T cells, mercury-treated DP rats do not experience major changes in peripheral lymphocyte subsets or numbers. There were also no mercury effects on thymocytes of DP rats from Group BB#1, similarly to what occurs in LEW rats and in contrast to the thymic atrophy observed in mercury-treated BN rats.[19] However, DP rats of Group BB#3 treated with $HgCl_2$ for a protracted period of time had significant increases in thymocyte numbers when compared to controls, even though there was no apparent correlation between numbers of thymus cells and levels of autoantibodies to laminin and thyroglobulin.

Our experiments show that neither a brief or a long exposure to mercury inhibited autoimmunity in BB rats. One possible explanation for the absence of this effect could be that the first mercury injections were administered after the diabetogenic process had already started, even if the animals had no glycosuria at that time. This possibility might be correct for rats of Group BB#1, that were 74–87 days of age at the beginning of the experiment. On the other hand, rats of Groups BB#2 and BB#3 were much younger (32–35 day-old) when first injected with mercury. Animals of that age have no insulitis, a pathological process that precedes the appearance of glycosuria.[33] Experimental manipulations of 22–65 day-old DP rats (e.g., adoptive transfer of "normal" spleen cells or CD4+ T lymphocytes) completely inhibit both insulitis and clinical IDDM and have suggested the existence of a critical developmental period for IDDM from approximately 30 to 60 days of age.[51,52] Therefore, the possibility that the triggering event for autoimmunity preceded the timing of mercury administration may be rather remote. However, we are currently investigating possible effects of mercury in suckling DP rats. Alternatively, the difference between the immunosuppressive action of mercury on experimentally induced autoimmunity in LEW rats and its absence in BB rats might be due to the activation of CD8+ regulatory T cells by mercury in the former and not the latter. Numbers of CD8+ peripheral T lymphocytes are normal in LEW and deficient in DP BB rats.[55–55] However, diabetes is inhibited by CD4+, not CD8+ T cells in DP rats.[13,52,56] Similarly, in irradiated, thymectomized PVG rats it is a CD4+, not a CD8+ T cell that prevents autoimmunity.[57] Preliminary experiments ongoing in our laboratory also suggest that the lack of inhibitory effects of mercury is not due to the deficiency of CD8+ T lymphocytes observed in DP rats. DR rats have CD8+ T cells, but IDDM can be induced in these animals by combined depletion of RT6+ T lymphocytes and administration of poly-I:C. Mercury treatment does not inhibit IDDM even in these animals (Kosuda et al., manuscript in preparation).

In conclusion, the inhibitory activity of $HgCl_2$ on autoimmunity may be a unique strain-dependent characteristic of LEW rats. We know that the autoimmune consequences of mercury exposure are genetically controlled and vary with inbred strain of rats. Exposure to $HgCl_2$ in BN, MAXX and DZB rats results in the production of autoantibodies to laminin and other autoantigens, whereas similarly treated PVG rats produce antinuclear antibodies.[43] In contrast, LEW rats respond to mercury treatment by developing immunosuppression, a possibility confirmed by the observation that $HgCl_2$-treated F1 hybrids between BN and LEW are protected against the development of experimentally induced uveoretinitis or pinealitis.[50,58] This protection is not mediated by CD8+ T suppressor cells and may involve a lack of balance between Th2 and Th1 cells.[47,48,50,59] Indeed, differences in regulatory T cell subsets and/or the cytokine networks may be responsible for variations in both autoimmune and

immunosuppressive effects of mercury between rat strains, a hypothesis currently investigated in our laboratories. Extension of the potential immunosuppressive effects of mercury on autoimmunity may be dependent on the subset of cells that can modulate the activity of the autoreactive cell population. Therefore, it should be cautioned that knowledge of the immunomodulatory mechanisms of each individual autoimmune disorder may be required prior to utilization of xenobiotics that mimic mercury effects as therapeutic immunosuppressive agents.[10,11]

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Abbreviations

ANOVA, analysis of variance; BN, Brown Norway; bw, body weight; DIF, direct immunofluorescence; DP, diabetes-prone; DR, diabetes-resistant; GBM, glomerular basement membrane; IDDM, insulin-dependent diabetes mellitus; LEW, Lewis; "M", $HgCl_2$-treated rats; SD, Sprague Dawley; TBM, tubular basement membrane; VAF, viral antibody-free; "W", H_2O-treated control rats.

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