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Lack of graft-versus-host-like pathology in mercury-induced autoimmunity of Brown Norway rats

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Abstract

The repeated administration of mercury to Brown Norway (BN) rats induces the production of autoantibodies to laminin 1 and other autoantigens, accompanied by renal deposition of immunoglobulins and a membranous glomerulonephropathy. A graft-versus-host-like (GVHL) syndrome, characterized by widespread necrotizing leukocytoclastic vasculitis of the bowel, skin, and other tissues, has also been observed after mercury treatment of BN rats. These findings have suggested that the autoimmunity caused by the administration of mercury to BN rats may result as a xenobiotic-induced GVHL effect under the control of OX22+ T lymphocytes. However, previous studies of mercury-induced autoimmunity have never reported any evidence of GVHL lesions. Therefore, we have carefully examined various tissues from a large group of BN rats injected with $HgCl_2$ to identify possible areas of inflammatory reactions that may have been unnoticed in previous investigations. In addition, we have determined by flow cytometry whether exposure to mercury results in percentage and numerical alterations of OX22+ or other lymphocyte subpopulations in lymphoid organs of $HgCl_2$-treated BN rats. The present article confirms that mercury induces autoimmune responses to laminin 1 but does not corroborate the hypothesis of a GVHL syndrome regulated by OX22+ lymphocytes. First, changes in OX22+ cells during treatment with $HgCl_2$ were infrequent and had no significant correlation with the kinetics of autoimmune responses to laminin 1. Second, we detected no GVHL lesions in skin and intestine of mercury-treated BN rats.

Keywords: Mercury; Autoimmune disease; Xenobiotics; Graft-versus-host-like pathology; Autoantibodies to laminin 1

Introduction

The repeated administration of low doses of mercury to Brown Norway (BN)[1] rats induces the production of autoantibodies to laminin 1 and other autoantigens, accompanied by renal deposition of immunoglobulins and a nephrotic syndrome (reviewed in [1]). A graft-versus-host-like (GVHL) syndrome has also been observed after mercury treatment of BN rats [2–7]. This syndrome, characterized by widespread necrotizing leukocytoclastic vasculitis of the bowel, skin, and other tissues, was exacerbated by in vivo administration of monoclonal antibody (mAb) OX22. This mAb reacts with the CD45RC isoform of CD45 on T-helper lymphocytes (comprising both $CD45RC^{high}$ and $CD45RC^{low}$ cells), CD8+ T lymphocytes and B cells [2,8,9]. These findings have suggested that mercury-induced autoimmunity of BN rats may represent a GVHL syndrome driven by an increase in Type 2 cytokines produced by CD4+ $CD45RC^{low}$ cells [2,4]. However, previous studies of mercury-induced autoimmunity have never reported any evidence of GVHL pathology. An early publication by Druet's group carefully described the presence of extrarenal immune deposits but detected no major pathological lesions by light microscopy [10]. Similarly, in our earlier investigations of mercury-induced autoimmunity we never observed any macroscopic or microscopic evidence of

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[1] Abbreviations: ANOVA, analysis of variance; BN, Brown Norway; bw, body weight; DIF, direct immunofluorescence; DP, double positive; FCM, flow cytometry; FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane; GVHL, graft-versus-host-like; IF, immunofluorescence; IgG, immunoglobulin G; PE, phycoerythryn; SA, streptavidin; SP, single positive; TCR, T-cell receptor.

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GVHL injury. In the present study we have carefully examined various tissues from a large group of BN rats injected with $HgCl_2$ to identify possible areas of inflammatory reactions that may have been missed in our previous investigations. In addition, we have performed flow cytometry (FCM) analysis to determine whether exposure to mercury results in percentage and numerical alterations of OX22+ or other lymphocyte subpopulations in lymphoid organs of $HgCl_2$-treated BN rats. The present article confirms that mercury induces autoimmune responses to laminin 1 but does not corroborate the hypothesis of a GVHL syndrome regulated by OX22+ cells. First, changes in OX22+ and OX22− T cells were rare and had no significant correlation with the kinetics of $HgCl_2$-induced autoimmune responses to laminin 1. Second, we detected no GVHL lesions in skin and intestine of mercury-treated BN rats.

Materials and methods

Experimental animals

A total of 133 BN rats (female and male, divided in groups of different age and body weights, ranging from 95 to 400 g) were obtained from two commercial sources (Harlan Sprague–Dawley, Indianapolis, IN and Charles River Laboratories, Wilmington, MA). All animals were housed in plastic microisolator cages with wood shavings in an automated light cycle environment (12:12 h) and received standard autoclaved rat chow and sterile water ad libitum. All rats were certified by the vendor to be free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H1 (Toolan's virus), GD7 (mouse poliovirus), Reo-3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, *Encephalitozöon cuniculi*, and pinworm. Rats were housed in a viral-antibody-free facility, and monthly testing of sentinel rats was used to assure the absence of infection in experimental animals. All rats were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School and of the University of Connecticut Health Center and recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Administration of mercury

Rats were anesthesized prior to treatment. They were injected every other day subcutaneously (sc) or intraperitoneally (ip) with 0.1 ml of a mercuric chloride solution ($HgCl_2$, 1 mg/ml) per 100 g body wt, as previously described [11]. Rats were sacrificed at different intervals during treatment, as follows: 2–4 days (one to two injections = total dose of 100–200 μg $HgCl_2$/100 g body wt/rat), 5–7 days (three injections = total dose 300 μg/100 g), 8–9 days (four injections = total dose 400 μg/100 g), 10–12 days (five injections = total dose 500 μg/100 g), 14–16 and 21–30 days (six injections = total dose 600 μg/100 g). Control rats, of same age and weight as those injected with $HgCl_2$, were injected sc with distilled water at pH 4.2 following the same schedule.

ELISA for serum autoantibodies to laminin 1

Circulating autoantibodies to laminin 1 were detected by ELISA as previously described [12]. In brief, sera from mercury-treated and water treated BN rats were incubated in ELISA plates that had been previously coated with mouse laminin 1 (1 μg/100 μl/well), obtained courtesy of Dr. Hinda Kleinman (NIH, NIDR) [13,14]. All sera were tested in duplicate wells using 1:100 dilutions. After 30 min of incubation and washing, 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) was used to detect the binding of rat antibodies to laminin 1. ABTS substrate was added and ELISA plates were read at OD_{405} in a Thermomax ELISA reader (Molecular Devices Corporation, Menlo Park, CA 94025) at intervals of 10, 15, and 20 min of incubation with the substrate at room temperature. The "positive/negative cutoff" line was determined as previously described by calculating the mean OD_{405} + 3 SD of reference sera [12]. Negative reference sera were obtained from BN rats before the start of mercury treatment and from water-treated rats. Positive control sera for antibodies to laminin 1 were obtained from mercury-treated BN rats examined in previous studies [12].

Flow cytometry (FCM) for surface phenotype of rat lymphocytes

Spleen, lymph node, and thymus cell suspensions were obtained by gently pressing the tissues through a stainless steel screen (50 mesh), as previously described [12]. The six proximal cervical lymph nodes were recovered for total cell counts and subset analysis in all experiments. Cells were washed in two changes of cold medium (RPMI 1640) and cell viability determined was by trypan blue exclusion. The following mouse monoclonal antibodies (mAb) to rat cell surface markers were used: R7.3 (anti-TCR$\alpha\beta$), OX19 (anti-T), W3/25 or OX35 (anti-CD4), OX8 (anti-CD8), OX22 (anti-CD45), 10/78 (anti-NKR.P1A), and OX33 (anti-B cell). Rat mAb 6A5 (anti-ART2.2) and DS4.23 (anti-ART2.1) were used for detection of RT6+ T cells. Different unconjugated, biotinylated, or fluorochrome-conjugated mAb were used in one-, two- or three-color immunofluorescence (IF). Nonspecific antibody binding was determined using appropriate combinations of isotype-matched irrelevant mAb and secondary reagents. For one-color IF, cell suspensions were usually stained by direct IF with mAb to rat cell surface markers (CD5, CD4, CD8, TCR$\alpha\beta$, etc.), conjugated with either fluorescein isothiocyanate (FITC) or phycoerythryn (PE). Alternatively, indi-

Table 1
BN rats injected with $HgCl_2$ experience autoimmune responses to laminin 1 and show in vivo bound immune deposits but lack graft-versus-host-like (GVHL) pathology

Total amounts of $HgCl_2$ injected (μg/100 g body wt/rat)	Days of sacrifice	Percentage of BN rats with		
		Circulating antibodies to laminin 1	In vivo bound immunoglobulins[a]	GVHL pathology[a]
100–200	2–4	0	0	0
300	5–7	20	27	0
400	8–9	70	100	0
500	10–12	100	100	0
600	14–16	100	100	0
600	21–30	100	100	0

[a] See details under Results and in Table 4.

rect IF was performed with unlabeled mAb and developed with an $F(ab')_2$ 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. Staining for ART2+ cells was done by direct IF with anti-ART2.2 or anti-ART2.1 rat mAb, conjugated with fluorescein isothiocyanate (FITC) or phycoerythryn (PE). Controls routinely included the FITC conjugate alone and irrelevant rat and mouse monoclonal antibodies. For two-color IF, different combinations of mAb conjugated with FITC and biotinylated mAb plus PE-labeled streptavidin (SA) were used. For viability studies a sample of unfixed cells was examined by trypan blue or propidium iodide. Otherwise, cell suspensions were fixed after staining and before FCM using a 1% EM-grade paraformaldehyde solution in PBS. FCM analysis was performed using a B-D FACScan equipped with an argon-ion laser light source and CellQuest software. Dead cells and contaminating red blood cells were excluded from analysis by electronic gating. A total of 10,000 nucleated cells were analyzed for relative fluorescence intensity. The monoclonal antibodies used for this study have been previously described and are commercially available, except for the rat mAb anti-ART2.2 and anti-ART2.1, that are both obtained from hybridomas cultured in our laboratory [11,12,15,16].

Immunohistopathology of rat kidneys and other tissues

Demonstration of rat immunoglobulin G (IgG) bound to rat tissues was performed by direct immunofluorescence (DIF) as previously described [15]. Cryostat sections of rat kidney, intestine, skin, thyroid, liver, parotid, pancreas, lung, etc. were stained with FITC-conjugated rabbit antibodies to rat IgG (Jackson ImmunoResearch Laboratories, Inc.) and read using a fluorescence microscope equipped with epi-illumination (Leitz Dialux, E. Leitz Inc., Rockleigh, NJ). Duplicate sections from each rat were examined and defined as positive when linear staining of IgG was present at the level of the basement membranes. The intensity of DIF staining in each 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.

Histopathology

Samples of kidney, thyroid, pancreas, intestine, skin, liver, parotid, lung, etc. from mercury-treated and control BN rats were fixed in buffered formalin and embedded in paraffin and sections were stained with H&E. Separate samples from the same tissues were embedded in plastic (JB-4 medium, Polysciences Inc., Warrington, PA) and sections cut at 3 μ (Sorvall JB-4 microtome, Dupont Instruments, Newtown, CT). Each section was stained with toluidine blue and a duplicate section with H&E or DiffQuik (American Scientific Products). All sections were examined by light microscopy.

Statistical evaluation of data

All ELISA and DIF tests were performed in duplicate and the data obtained from all experimental and control groups expressed as the mean ± standard error of the mean (SEM). FCM analysis was performed on single samples from each rat and the data from all groups presented as the mean ± SEM. 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, Games–Howell and Dunnett's multiple comparison procedures). The StatView 4.0 program for Macintosh computers (Abacus Concepts, Inc., Berkeley, CA) has been used for this purpose.

Results

Kinetics of autoimmune responses to laminin 1 induced by mercury treatment of BN rats

Sera from a total of 85 BN rats treated with $HgCl_2$ were tested by ELISA for antibodies to laminin 1. None of 12 rats that were sacrificed on days 2–4 of treatment and therefore had received a total of only 100–200 μg $HgCl_2$/100 g body wt/rat had developed antibodies to laminin (Table 1). On the

Table 2
Kinetics of effects of mercury treatment on percentages of ART2.2+ and OX22+ lymphocytes[a]

Treatment	Day of sacrifice (and total amounts of $HgCl_2$ injected[b])	ART2.2+ (single positive[c])	TCR-OX22+ (single positive[d])	TCR+ OX22+ (double positive[e])	TCR+ OX22- (single positive[f])
H_2O		37 ± 7	35 ± 1	9 ± 1	49 ± 0.3
$HgCl_2$	4 (200)	36 ± 1	34 ± 3	17 ± 5	43 ± 3
	8 (400)	31 ± 3	39 ± 2	10 ± 3	38 ± 4
	11 (500)	**9 ± 6**	29 ± 4	**23 ± 7**	**22 ± 5**
	15 (600)	**14 ± 9**	26 ± 14	5 ± 1	36 ± 5

[a] A total of 11 $HgCl_2$-treated and 11 H_2O-treated BN rats were sacrificed at different days during treatment. Data are expressed as mean percentage (±SEM) of positive cells from six cervical lymph nodes. In bold are percentages that reached statistical significance (see Results for *P* values).
[b] In brackets are the total amounts injected=μg $HgCl_2$/100 g bw/rat.
[c] ART2.2 single positive detected by FITC.
[d] OX22 single positive detected by PE.
[e] Double positive for TCR and OX22, detected by FITC and PE.
[f] TCR single positive detected by FITC.

other hand, positive reactions were obtained with sera from 3 of 15 rats sacrificed on days 5–7 (20%) and 7 of 10 sacrificed on days 8–9 (70%). All sera obtained after day 10 of treatment (6/6 rats for days 10–12, 38/38 for days 14–16, and 4/4 for days 21–30) were found to be positive for antibodies to laminin 1. As shown in Table 1, the increase in percentage of positive animals was correlated with time of treatment and total amounts of mercury received by each rat. Serum levels of antibodies to laminin 1 also increased with time, as indicated by mean ELISA OD_{405} (data not shown). Sera from a total of 46 control BN rats, that had been injected with water at the same pH of the mercury solutions and were sacrificed at different times (3 on days 2–5, 9 on days 6–9, 34 on days 11–16) contained no antibodies to laminin 1.

Changes in OX22+ lymphocytes do not correlate with autoimmune responses to laminin 1

In this experiment we performed one-color and two-color IF to detect the kinetics of possible changes in lymphocyte subpopulations obtained from the six proximal cervical lymph nodes of BN rats treated with mercury and sacrificed at different times of treatment. Table 2 provides the most relevant FCM data from cells expressing ART2.2, OX22, and/or TCR. Results of FCM for other surface markers (CD4, CD8, etc.) provided no additional information as compared to our previously published data and are not shown for the sake of brevity. As we previously reported [15,17], there was a decrease in the percentage of ART2.2+ lymphocytes on D11 ($P = 0.077$) and D15 ($P = 0.0400$). The percentage of OX22+ (single positive, TCR− OX22+) cells of BN rats injected with $HgCl_2$ was similar to that of water-treated controls on D4 ($P = 0.8509$), D8 ($P = 0.4816$), and D11 ($P = 0.2844$). It was slightly decreased on D15 ($P = 0.1724$ vs controls; $P = 0.2616$ vs D4; $P = 0.0811$ vs D8; $P = 0.6704$ vs D11). The percentage of OX22+ T lymphocytes (double positive, TCR+ OX22+) increased on D4 ($P = 0.1333$ vs controls), was unchanged on D8 ($P = 0.8647$), slightly increased on D11 ($P = 0.0184$), and decreased slightly on D15 ($P = 0.4961$). Variations of TCR+ (single positive, TCR+ OX22−) cells were not significant except for a day 11 decrease ($P < 0.0001$ vs controls). Thus, changes in OX22+ lymphocytes and OX22+ T cells did not occur consistently during mercury treatment and when present had no significant correlation with the kinetics of autoimmune responses to laminin 1.

Initial changes in lymphocyte subpopulations of mercury-treated BN rats

We then evaluated by FCM whether other lymphocyte subsets exhibited any changes in the initial stages of exposure to mercury. As shown in Table 3, four BN rats that had been injected only 3 times (for a total of 300 μg/100 g bw) and were sacrificed on D6 of treatment experienced a modest increase in the total number of cervical lymph node cells when compared to four H_2O-treated controls of the same group. They also had a modest reduction in ART2.2+ cells, similar to the changes described in Table 2 but less significant than the decrease previously observed by our group in younger mercury-treated BN rats [15]. Higher levels of ART2+ cells are usually detected in older rats. There were also variations in percentages of NKR+ and NKR+TCR+ cells. When tested by ELISA, sera of these four BN rats contained no autoantibodies to laminin 1. Direct IF of their kidneys did not show any immunoglobulin binding to renal structures. Thus, at a time when other subsets of lymphocytes, including CD4+ and CD8+ T cells (data not shown), appeared unaffected and there were no detectable autoimmune responses to laminin, there was a significant increase in both percentage and absolute numbers of both NK cells and NK T lymphocytes.

Immune deposits in kidneys and other tissues

Table 1 shows the results obtained by DIF for immunoglobulin deposits in tissues of 87 $HgCl_2$-treated BN rats. No

Table 3
Changes in lymphocyte subpopulations of BN rats sacrificed on day 6 of mercury treatment

Treatment[a]	Number of cells ($\times 10^6$)[b]	Percentage ART2.2+	Percentage TCR+	Percentage NKR+	Percentage NKR.P1+TCR+
H_2O	18 ± 3	64 ± 3	70 ± 2	1.8 ± 0.2	3.2 ± 0.1
$HgCl_2$	63 ± 13	53 ± 2	62 ± 2	2.7 ± 0.1	5.3 ± 0.5
P	0.0154	0.0396	0.0312	0.0080	0.0044

[a] Four BN rats were injected with $HgCl_2$ (100 μg/100 g body wt) every other day for a total of three injections (total amounts 300 μg/100 g body wt). As controls, four BN rats were injected with H_2O following the same schedule. All animals were sacrificed on day 6 of treatment.
[b] Cells obtained from six cervical lymph nodes.

such deposits were observed in 10 rats sacrificed on days 2–4. They were first observed in 4/15 (27%) BN rats after 5–7 days of treatment. The percentage of positive animals increased to 100% in animals sacrificed after 8 days (9/9 on days 8–9, 3/3 on days 10–12, 44/44 on days 14–16 and 6/6 on days 21–30). Control BN rats injected with water had no tissue-bound immunoglobulin deposits. As previously described [12], all positive rats had immunoglobulins bound to their kidneys, with a linear distribution on renal glomerular basement membranes and often tubular basement membranes. In addition, all other tissues examined (skin, intestine, liver, parotid, lung, thyroid, pancreas, tongue, esophagus, stomach, heart, uterus, ovary, adrenal, testis, vas deferens) had linear immunoglobulin deposits at the basement membrane level (Table 1).

Mercury-treated BN rats showed no evidence of graft-versus-hostlike pathology

There were no macroscopic alterations in skin or other tissues. In particular, the skin of mercury-treated BN rats did not show ulcerations of surface epithelium. Similarly, the macroscopic appearance of the intestinal cecal serosa and mucosa was normal. The lumen of the intestine was not edematous and lacked petechial or severe hemorrhagic lesions. We then screened various organs and tissues for possible evidence of GVHL histopathology induced by mercury exposure (Table 4). In addition to H&E-stained sections we examined 3-μ-thick glycolmethacrylate-embedded sections that provide a high resolution of tissue structures and a more accurate visualization of histopathology than paraffin-embedded sections [18]. The skin of 22 mercury-injected BN rats did not show polymorphonuclear or mononuclear cell inflammation. This group of rats comprised 18 animals that had received the full mercury treatment (total dose 600 μg/100 g body wt/rat), 2 rats that had been injected with a total of 400 μg/100 g body wt/rat, and 2 rats injected with a total of 300 and 100 μg/100 g body wt/rat each. We also examined the intestine of the same group of 22 rats (plus 1 additional animal that had received the total dose of 600 μg/100 g body wt) and found that it was normal. There were no signs of "early vasculitis" after 2–5 days or "late vasculitis" after 12–20 days of mercury treatment. We detected no leukocytic infiltration of the lamina propria of the mucosa and serosa of the duodenum, no necrotizing vasculitis of submucosal vessels in the colon, and no mononuclear cell infiltrate close to those vessels. Thus, two major target organs of graft-versus-host disease showed no signs of cell-mediated lesions. However, both experimental and control rats of this group had inflammatory infiltrates in other tissues. Histopathologic signs of acute and chronic inflammation were detected in liver (4/22

Table 4
Histopathology of mercury-treated and control BN rats

Treatment	Tissue	Number of BN rats with inflammation	Description of histopathology[a]
$HgCl_2$	Skin	0/22	Normal skin
	Intestine	0/23	Normal intestine
	Thyroid	2/54	Chronic inflammation
	Pancreas	4/15	Chronic inflammation
	Liver	6/22	Acute & chronic inflammation
	Lung	16/19	Acute & chronic inflammation
H_2O	Skin	0/10	Normal skin
	Intestine	2/10	Small mononuclear cell infiltrates
	Thyroid	1/18	Chronic inflammation
	Pancreas	3/11	Chronic inflammation
	Liver	6/10	Acute & chronic inflammation
	Lung	7/7	Acute & chronic inflammation

[a] Data obtained from various groups of BN rats that received from 100 to 600 μg $HgCl_2$/100 g body wt/rat or control animals injected with H_2O. See Results for details.

mercury-treated and 6/10 controls) and lung (16/19 mercury-treated and 7/7 controls). The lung lesions were characteristic of interstitial pneumonitis and comprised polymorphonuclear neutrophil leukocytes, eosinophil leukocytes, and/or mononuclear cells. We also examined the thyroids of this group and an additional 15 rats (3 injected with a total dose of 200, 3 with 300, 3 with 400, 3 with 500, and 3 with 600 μg/100 g body wt, respectively). Chronic inflammation was present in the thyroids of only 2/54 BN rats injected with $HgCl_2$ and 1/18 water-treated controls. The pancreas and peripancreatic tissues of 4/15 mercury-treated and 3/11 control animals also showed chronic inflammation.

Discussion

Exposure to mercury may result in autoimmune responses and disease in humans, a phenomenon that highlights the important health risks of some environmental chemicals (reviewed in [1]). These effects have been investigated by the experimental administration of $HgCl_2$ to rabbits, mice, and rats. Depending on animal species, different types of autoimmune responses are observed after treatment with mercury. Some strains of mice (C57BL/6J, BALB/c, AKR) produce autoantibodies that react only with nuclear antigens [19–23] and have scarce or absent renal immune deposits similarly to human subjects with drug-induced lupus. Other mouse strains (SJL/J, B10.S) produce high levels of autoantibodies to nucleolar "fibrillarin" (U3 RNP protein) [19], resembling the serologic findings observed in a subset of scleroderma patients. In Brown Norway rats mercury induces autoantibodies to laminin 1, renal IgG deposits, and a nephrotic syndrome [1]. Our present survey of a large number of BN rats injected with $HgCl_2$ or H_2O confirms previous reports that autoimmune responses to laminin 1 are induced by mercury treatment and are correlated with total amounts of xenobiotic administered [15,24,25]. Our examination of a variety of tissues stained by DIF for IgG deposits has also validated earlier literature reports. Both renal and extrarenal linear IgG deposits have been previously described in the literature [1,10,26]. Complement was not detectable in immune deposits bound to renal GBM and there were no inflammatory changes in glomeruli [27,28]. As shown in Table 1, kidneys of 100% of mercury-treated BN rats sacrificed on days 8–30 had linear immunoglobulin deposits at the GBM level and presented the histopathologic pattern of a membranous nephropathy (i.e., noninflammatory lesions). IgG deposits with a linear distribution were also present in a variety of tissues.

Contrary to previous reports [4,8] we observed that variations in OX22+ cells (a possible immunoregulatory subpopulation) do not correlate with the kinetics of mercury-induced autoimmune responses. As demonstrated in Table 2, there were no significant changes in percentages of single positive OX22 cells over time. Similarly, there was no consistent variation of T cells expressing OX22, i.e., double-positive TCR+ OX22+ lymphocytes. The difference between our data, obtained from cervical lymph nodes, and those reported by authors using mesenteric lymph nodes [4] may be due to the source of the lymphocytes tested by FCM for surface markers. OX22+ cells may be changing in mesenteric lymph nodes and not in cervical lymph nodes, with variations caused by migration patterns, integrins, local cytokine production, and go on. This possibility is not favored by the observation that standard mercury treatment causes a generalized hyperplasia of spleen and lymph nodes, suggesting an equal involvement of all lymphoid sites without evidence of a regional effect [29]. However, the issue can only be solved by a detailed comparative analysis of FCM changes in different lymph nodes that we will perform in future investigations.

OX22+ T cells are of great interest, because Mason's group suggested that $OX22^{high}$ CD4+ T lymphocytes might be Th1-like and $OX22^{low}$ CD4+ T lymphocytes Th2-like [30]. However, most OX22+ cells are not contained in the T-cell population and in other experiments (data not shown) we have observed that the majority are actually B cells. In conclusion, our FCM data suggest that OX22+ cells may not have an important regulatory role in mercury-induced autoimmunity of BN rats. It is still unclear whether such a role is exercised by other lymphocyte subpopulations. We have previously shown that ART2.2+ cells decrease during mercury treatment of BN rats [15], a finding confirmed in the present study (Tables 2 and 3). ART2 T lymphocytes are one of various putative immunoregulatory subpopulations [31,32]. As shown in Table 3, we have also noticed an early increase in NK and NK T lymphocytes at a time (day 6) when circulating autoantibodies to laminin 1 were not detectable in the 4 rats tested. This may be an intriguing finding that deserves additional investigations in view of the recent interest in modulatory functions of NK cells [33,34].

A graft-versus-host-like (GVHL) syndrome has been described in BN rats injected with mercury [2–6]. This syndrome was characterized by widespread necrotizing leukocytoclastic vasculitis of the bowel, skin, pancreas, liver, lung, and a variety of other organs. Those publications specified that all $HgCl_2$-treated BN rats developed severe inflammation and ulceration of the skin (perioral skin, footpad) by day 14 of treatment. Microscopic examination of these areas revealed ulceration of surface epithelium with underlying mononuclear cell inflammation (subepidermal and perihair follicle lymphocytic infiltration). In addition, mercury-treated BN rats developed vasculitis of the bowel in a biphasic pattern [35]. A first phase (early vasculitis) started 1–5 days after the initiation of treatment (i.e., after two injections). A second phase (late vasculitis), characterized by more severe tissue injury, occurred between 12 and 20 days after five to six injections. The duodenum showed prominent foci of intense leucocytic infiltration of the lamina propria and serosa [2]. In the colon there was severe necrotizing vasculitis of submucosal vessels with moderate

to severe mononuclear cell infiltrates close to vessels. The liver contained portal collections of large activated lymphocytes. The pancreas had peripancreatic mononuclear cell infiltration with occasional heterophil leukocytes. It was also reported that the lungs of one control animal and all mercury-treated rats had mild interstitial pneumonitis, characterized by heterophil leukocytes and lymphocytic infiltration. These findings were interpreted as confirming the old hypothesis that xenobiotic-induced autoimmune responses resemble graft-versus-host reactions [36–38].

Because our own experience did not agree with those observations, we have carefully examined paraffin-embedded H&E-stained as well as plastic-embedded toluidine-blue-stained thin sections of tissues from a large number of mercury-treated and control BN rats (Table 4). We detected no histopathologic alterations in the skin (0%) and intestine (0%) of these animals. Gross examination of these tissues had also shown no lesions. Thus, two primary target organs for cell-mediated immune damage during graft-vs-host disease [39–41] were found to be completely normal. On the other hand, acute and/or chronic inflammation was present in other tissues from *both* control and experimental rats. The thyroids of 6% control and 4% mercury-treated BN rats showed some areas of chronic inflammation, with no visible differences between the two groups of animals. The pancreas was similarly affected (27% in both experimental and controls). Signs of acute and chronic inflammation were detected more frequently in livers from control rats (60%) than from mercury-treated animals (27%). The lungs of control (100%) and mercury-injected (84%) BN rats were particularly involved by acute and chronic inflammatory processes, suggesting that a subclinical microbial infection was occurring in the respiratory tract and other organs of all these animals.

The difference between our results and those described previously by other authors is difficult to explain. The great majority of our BN rats received mercury subcutaneously in standard doses identical to those used in the reports describing GVHL pathology. This excludes possible variations due to dosage and route of administration of mercury. On the other hand, there might be strain differences between the BN rats we have used and those reported to exhibit GVHL lesions. This possibility is suggested by changes in the incidence of autoimmune responses and disease in sublines of inbred rats and mice [42–45]. However, one publication by Mathieson's group stated that similar GVHL findings were obtained with BN rats from two different commercial sources and their own established colony, thus casting some doubts on the strain difference hypothesis [2]. In our opinion, the most likely explanation may be provided by the same report showing that the inflammatory changes in various tissues disappeared after antibiotic treatment [2]. Thus it is possible that inflammation resulted as a consequence of synergistic effects of mercury and microorganisms in rats that were not specific-pathogen-free. Occasional outbreaks of infection do occur in experimental animals, even when they are obtained from reliable suppliers and despite the best precautionary measures. An infection, possibly aggravated by immunotoxic effects of mercury and/or the stress of anesthesia and repeated injections of mercury or distilled water, may have been responsible for diffuse inflammatory reactions that appeared as the induction of a GVHL syndrome. This possibility is confirmed by our finding of acute and chronic inflammatory changes in the lungs of control as well as mercury-treated rats.

In conclusion, our study shows that autoimmune responses induced by administration of mercury to BN rats are not associated with inflammatory changes of skin and intestine and do not seem to result as a xenobiotic-induced GVHL effect. This is not surprising because mercury can cause autoimmunity through the direct stimulation of a variety of lymphocyte subpopulations involved in the induction and effector phases of the immune response [1]. It can also inhibit immunoregulatory cells. These effects resemble the activity of infectious microorganisms, another possible cause of autoimmune disease [32,46,47]. They may be mediated by interactions with pattern recognition receptor molecules present on cells of the immune system. They can also be caused by reactions with cellular protein thiols, modifications of host tissue epitopes as well as induction of cell death by apoptosis and/or necrosis. In summary, numerous and diverse mechanisms likely result in autoimmunity induced by mercury. Therefore, investigations of immune cells and molecular pathways involved in the autoimmune effects of this metal have considerable interest for a deeper understanding of xenobiotic-induced and idiopathic autoimmune disease.

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