Mercury-induced Renal Autoimmunity: Changes in RT6+ T-Lymphocytes of Susceptible and Resistant Rats

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Recent studies from our laboratory have shown that treating BN rats with HgCl₂ causes a decrease in peripheral RT6+ T-lymphocytes, a change that coincides with the appearance of circulating autoantibodies to renal antigens (17). We have proposed that autoimmune kidney disease induced by mercury in genetically susceptible rats may be a consequence of interactions between this metal and a subset of regulatory (RT6+?) T-cells. In contrast, Lewis (LEW) rats are resistant to the autoimmune effects of mercury and reportedly develop a subpopulation of suppressor T-lymphocytes (18,19). Therefore, it was of interest to compare the effects of mercury on lymphocyte subpopulations from BN, LEW, and (BN × LEW) F₁ rats. The hybrids between the mercury-susceptible BN and the mercury-resistant LEW strain are susceptible to the autoimmune effects of HgCl₂, a trait inherited in a dominant fashion (4). We show here that LEW rats injected with HgCl₂ do not have significant decreases in RT6+ T-lymphocytes or detectable autoimmune responses. Instead, both BN and (BN × LEW) F₁ hybrid rats experience autoimmune responses to renal antigens as well as similar changes in RT6+ T-cells after mercury treatment.

Methods

We obtained 107 female rats [39 LEW, 36 (BN), and 32 (BN × LEW) F₁ hybrids, hereafter referred to as F₁ hybrids] from a commercial source (Harlan Sprague Dawley, Indianapolis, IN). All animals 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. Treatment of all rats followed the standard National Institutes of Health guidelines: light ether anesthesia was used for all procedures, including bleeding from the retro-orbital plexus or exsanguination followed by transection of the thoracic aorta at the termination of the experiments.

Previous studies have shown that treatment with mercury in its various chemical forms, using different routes of administration (subcutaneous injection, oral ingestion, inhalation), results in autoimmune responses to a variety of antigens (7–10). Relatively low doses of mercury have been found to be effective in both rats and mice: for example, the immunotoxic effects of HgCl₂ occur in BN rats at levels (3–25 μg/100 g body weight) that are much lower than those causing the common nephrotoxic effects (11,12, Bigazzi et al., unpublished data).

When injected with HgCl₂, animals from the susceptible strains experience a membranous glomerulonephritis, characterized by proteinuria and the production of autoantibodies to epitopes of the renal glomerular basement membrane (GBM), including laminin (5,13–16). This disorder has a self-limiting course somewhat similar to that of monophasic experimental allergic encephalomyelitis (EAE) in rats: autoimmune responses reach their peak approximately 2 weeks from the beginning of mercury treatment, then regress spontaneously in spite of continuous administration of HgCl₂. BN rats that have recovered from this glomerulonephritis are subsequently resistant to additional treatment with mercury.

The repeated administration of mercury to rats of the Brown Norway (BN) inbred strain results in a self-limiting production of autoantibodies to renal antigens (e.g., laminin) and autoimmune glomerulonephritis. In contrast, rats of the Lewis (LEW) strain do not develop renal autoimmunity after mercury treatment. Suppressor T-cells and/or the idiotype-anti-idiotype network have been implicated in the control of autoimmunity in susceptible (BN) rats as well as the “resistant” state of nonsusceptible (LEW) animals. In our investigations of the immune regulation of mercury-induced autoimmune glomerulonephritis, we have performed a phenotypic analysis of lymphocyte subpopulations in the spleens and lymph nodes of mercury-treated and control LEW, BN, and (BN × LEW) F₁ hybrid rats. Of particular interest were RT6+ T-cells, a subpopulation of lymphocytes that may have immunoregulatory properties and show a relative decrease in mercury-treated BN rats concomitant with the development of autoimmune responses to renal autoantigens. LEW rats did not develop renal autoimmunity after mercury treatment and had no significant change in the ratio of RT6+ to RT6– T-lymphocytes.

Interestingly, the administration of mercury to (BN × LEW) F₁ hybrid rats caused effects similar to those observed in the BN strain. Autoimmune responses to antigens of the kidney coincided with a change in the balance within the RT6 cell population, which was altered in favor of T-lymphocytes that do not express the RT6 phenotype. These results suggest that both genetic background and immune regulatory networks (possibly acting through T-lymphocytes of the RT6 subset) may play an important role in the expression of autoimmunity after exposure to a xenobiotic such as mercury.

Key words: animal models, autoimmune disease, flow cytometry, immunoregulatory T-lymphocytes, mercury, phenotypic analysis, RT6+ T-lymphocytes, xenobiotic-induced autoimmunity. Environ Health Perspect 101: 178-185(1993)

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HgCl₂ occur in BN rats at doses (3-25 µg/100 g body weight) that are much lower than those causing the common nephrotoxic effects (11,12, Bigazzi et al., unpublished data). However, in the experiments reported here, we used higher levels of HgCl₂ (100 µg/100 g body weight) to replicate the doses used in previous studies of BN and LEW rats (17,18,20).

BN, LEW, and F₃ rats were randomly divided into experimental and control groups and treated with mercury or water, respectively, as previously described (17). We injected 80 experimental rats (31 LEW, 25 BN, and 24 F₃ hybrids) subcutaneously on the abdomen with 0.1 ml of a mercuric chloride solution (HgCl₂, 1 mg/ml) per 100 g body weight. We dissolved HgCl₂ in distilled water and recorded the pH before use (range 3.9-4.2). Rats were injected under light ether anesthesia. We injected rats three times a week for 2 weeks, for a total administration of 600 µg HgCl₂/100 g body weight/rat. Four LEW rats died under ether anesthesia and one F₃ hybrid injected with mercury died on day 12 of mercury treatment: these animals are not included in the Results section. We anesthetized and injected 27 control rats (8 LEW, 11 BN, and 8 F₃, hybrid of same age and weight as those injected with mercury) with distilled water at pH 4.2 (acidified by adding 0.01 N HCl) following the same regimen.

ELISA for circulating autoantibodies to renal GBM and anti-laminin antibodies was performed as previously described (17). In brief, we incubated sera from mercury-treated and control rats in ELISA plates that had been coated previously with rat GBM or mouse laminin (500 ng/well). After incubation and washing, horseradish peroxidase-conjugated rabbit antibodies to rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used to detect the binding of rat anti-GBM to GBM (or anti-laminin to laminin). We read plates using a Titertek ELISA Reader at OD₄₅₄.

We determined rat IgG bound to renal GBM and tubular basement membrane (TBM) by direct immunofluorescence as previously described (17). In brief, cryostat sections of rat kidney were stained with fluorescein isothiocyanate (FITC)-conjugated rabbit antibodies to rat IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:20 in phosphate-buffered saline. We read the sections using a Leitz Dialux fluorescence microscope equipped with epi-illumination.

As previously described (17), spleen, lymph node, and thymus cell suspensions were obtained by gently pressing the tissues through a stainless-steel screen (50 mesh). The six proximal cervical lymph nodes were recovered for total cell counts and subset analysis in all experiments. We washed the cells in two changes of cold medium (RPMI 1640) and determined cell viability by trypan blue exclusion. Cell suspensions were labeled with anti-RT6.2 or anti-RT6.1 rat monoclonal antibodies (mAb) and developed for immunofluorescence with an F(ab')₂, fragment of an 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. When cells were labeled with mouse mAb directed against other cell surface markers (CD4, CD5, CD8, etc., see below), we developed them with an F(ab')₂, fragment of an 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 flow cytometry using saline-buffered 10% formalin and then analyzed using a fluorescence-activated cell sorter IV (Becton Dickinson, Mountain View, CA), according to the data of relative light scatter and relative fluorescence intensity as described previously (21,22). We excluded dead cells and contaminating red blood cells from analysis by electronic gating. At least 50,000 nucleated cells were analyzed for relative fluorescence intensity.

Hybridoma cell lines secreting mAb to the RT6.1 (clone DS4.23) and RT6.2 (clone 6A5) alloantigens are maintained in our laboratory (21,22). Clone DS4.23 was developed in the laboratory of Frank Fitch in collaboration with Lubaroff and Greiner (23). The 6A5 cell line was a gift of D.M. Lubaroff of the University of Iowa and was originally developed and characterized by B. Carpenter of Harvard University (24, 25). The RT6 alloantigenic system in the rat is not linked to the MHC and consists of two alloantigens, RT6.1 (expressed in rats of the Lewis and PVG strains) and RT6.2 (expressed in rats of the Brown Norway and Wistar Furth strains) (17). The RT6 antigen is expressed only by T-lymphocytes and approximately 45% of CD4⁺ and 70% of CD8⁻ peripheral T-cell subsets. No RT6⁺ thymocytes, bone marrow lymphocytes, or B-lymphocytes have been detected, nor has RT6 been identified on other tissues, including brain tissue. RT6⁺ T-cell subsets are severely decreased in diabetes-prone BB rats (i.e., animals that spontaneously develop insulin-dependent diabetes mellitus, both clinically and biochemically analogous to human diabetes mellitus) (26,27). Different lines of BB rats have been developed, one called “diabetes-prone” (DP) and one called “diabetes-resistant” (DR). In vivo depletion of RT6⁺ cells induces diabetes in 50% of DR BB rats (28). Lymphocyte transfers from DR to DP rats prevent diabetes mellitus, and this protection is associated with the persistence of donor-origin RT6⁺ T-cells (29). In addition, concanavalin A-activated spleen cells from RT6-depleted PVG or Yoshida rats can transfer insulin and/or thyroiditis to histocompatible athymic recipients (30).

We obtained mAb to additional rat lymphocyte markers from Seralab, Accurate Chemical & Scientific Corporation (Westbury, NY) and Harlan Bioproducts for Science, Inc. (Indianapolis, IN); MRC OX-19 (anti-CD5, pan-T-cells), R7.3 (anti-TCRβ), MRC OX-8 (anti-CD8 T-cell subset and natural killer cells), W3/25 (anti-CD4 T-cell subset and monocytes), MRC OX-39 (anti-IL-2 receptor), MRC OX-4 (anti-common class II MHC).

We obtained the total number of RT6⁺ lymphocytes from their percentage of the total cell numbers in spleen or lymph nodes. Then we calculated the total number of RT6⁺ cells by subtracting the total number of RT6⁺ lymphocytes from total T (CD5⁺) cell numbers. Finally, we obtained the RT6⁺ to RT6⁻ ratio by dividing the total number of RT6.1⁺ or RT6.2⁺ T-lymphocytes by the total number of RT6.1⁻ or RT6.2⁻ lymphocytes.

All ELISAs were performed in duplicate, and the data obtained are expressed as means ± SEM. Flow cytometric analysis was performed on single samples from each rat, and the data from all experimental (or control) groups are expressed as means ± SEM. We statistically evaluated results obtained by ELISA and flow cytometry by one-way analysis of variance (ANOVA), followed by post-hoc means tests (Fisher’s Protected Least Significance Difference, Scheffe’s, Games-Howell, and Dunnett’s multiple comparison procedures). Two computer programs (StatView II and SuperANOVA, Abacus Concepts, Inc., Berkeley, CA) were used for this purpose.

Results

Autoimmune Responses to Renal Antigens

We first examined the effects of mercury in two matching groups of LEW and BN rats, treated with mercury (“experimental”) or injected with water (“control”) and sacrificed on day 16 of treatment, i.e., the time when autoimmune responses to renal antigens are known to be at their peak in
BN rats (14, 17). None of the experimental LEW rats developed antibodies to laminin (Table 1) or to renal GBM (data not shown). On the other hand, approximately 86% of experimental BN rats produced antibodies to laminin (Table 1). Autoantibodies to rat GBM were also detected by ELISA in the sera of the same animals (data not shown). None of the control rats (LEW or BN) produced auto-antibodies to GBM or laminin. To ensure that the choice of the timing of observation (day 16) had no negative influence on our results, we performed a kinetic study in another group of LEW rats and sacrificed them at regular intervals (days 12, 16, 23, and 30) during their exposure to mercury. Again, none of these animals produced autoantibodies to GBM or laminin. For example, there were no statistical differences in the anti-GBM ELISA values obtained with sera from control (0.211 ± 0.021) or experimental rats (day 12 = 0.271 ± 0.114; day 16 = 0.235 ± 0.040; day 23 = 0.288 ± 0.104; day 30 = 0.238 ± 0.052). Instead, sera from experimental BN rats contained autoantibodies to rat GBM (0.606 ± 0.032). These findings confirm that rats of the LEW strain are resistant to mercury-induced autoimmunity, as previously reported by other investigators (4).

We then investigated the autoimmune consequences of exposure to mercury in F1 hybrid rats. Antibodies to laminin were present in the sera of 83% of experimental animals (Table 1). Autoantibodies against rat GBM were found in the same sera (data not shown). None of the control rats produced autoantibodies to GBM or laminin. These serological observations were confirmed by renal immunopathology data. Direct immunofluorescence of kidney sections revealed rat immunoglobulins bound in a linear fashion to renal GBM and TBM in 100% of experimental BN and F1 hybrids, whereas all experimental LEW rats gave negative results (Table 1). All control animals were also negative. Our observations confirm previous reports that rats from the BN strain and their F1 hybrids with resistant strains are susceptible to mercury-induced autoimmunity (4). Therefore, these animals provide an excellent model to determine possible changes in regulatory T-lymphocytes of the RT6 subpopulation.

**Phenotypic Analysis of Lymphocyte Subpopulations in LEW Rats**

Lymphocytes from spleens and cervical lymph nodes of experimental and control LEW rats, sacrificed at various intervals during treatment (days 12, 16, 23, and 30), were counted and examined by flow cytometry for a variety of surface markers, with the following results.

As shown in Table 2 and Figure 1, experimental LEW rats had a moderate but significant increase in numbers of spleen and lymph node cells. Higher spleen cell numbers were noted on days 12, 16, and 23 of treatment (93% in-cres, p = 0.0518, 86%, p = 0.0065 and 108%, p = 0.0289, respectively). Lymph nodes also contained higher numbers of cells on days 16 (232 increase, p = 0.0004) and 23 (46%, p = 0.0846).

There were no significant differences in percentage of RT6 cells from the spleens of control and experimental LEW rats on days 12, 16, and 23 (Table 2). Values for day 30 showed a moderate increase (26%, p = 0.0233). There were also no percentage changes in lymph nodes for days 12 and 30, but a slight decrease was observed on days 16 (12%, p = 0.0565) and 23 (16%, p = 0.0438). The total number of RT6.1 spleen lymphocytes showed a moderate increase on each day of treatment (day 12 = 92%, p = 0.0083; day 16 = 83%, p = 0.0045; day 23 = 112%, p = 0.0018; day 30 = 92%, p = 0.0083), whereas lymph node cells had a significant change only on day 16 (180% increase, p = 0.0001). The ratio of RT6 to RT6 cells did not usually vary in experimental animals versus controls, except for a modest increase (37%, p = 0.0186) observed in lymph node cells only on day 23. As shown in Figure 2, which compares values obtained from animals of different strains, there was no significant change in the RT6 ratio of LEW rats sacrificed on day 16.

The percentage of T-cells did not vary significantly in experimental versus control rats. Table 2 provides data obtained with mAb OX-19 (anti-CD5), but similar results were also obtained with mAb R73 (anti-TCRαβ). On the other hand, total numbers of T-cells had increases comparable to those observed in total cell numbers (data not shown). In the spleen, T-cell numbers were higher on days 12 (99%, p = 0.0442), 16 (65%, p = 0.0425), and 23 (107%, p = 0.0363). In lymph nodes they increased only on days 16 (224%, p = 0.004) and 23 (48%, p = 0.0763). Percentages of CD8 cells and CD4 lymphocytes varied little either in spleen or lymph nodes. Total numbers of CD8 T-cells did not change significantly in the spleen but were 258% higher in lymph nodes (p = 0.0009).
RT6+ T-lymphocytes did not change in a significant fashion in the spleen and changed only on day 23 in lymph nodes.

**Phenotypic Analysis of Lymphocyte Subpopulations in BN Rats**

We have previously reported that RT6+ T-lymphocytes from spleen and cervical lymph nodes of mercury-treated BN rats show a relative decrease, particularly evident in the ratio between RT6+ and RT6− cells (17). In the present series of experiments we examined the phenotypes of cells from a new group of experimental and control BN rats, sacrificed on day 16 (i.e., at the time when autoimmune responses to renal antigens reach their peak in animals injected with HgCl2), with the following results.

As shown in Table 3, the number of spleen cells in experimental BN rats was considerably higher than in controls, a difference that was statistically significant (218%, p = 0.0033). A more striking increase in total cell numbers (860%, p = 0.0001) occurred in cervical lymph nodes (Table 3), confirming previous observations of hyperplasia in peripheral lymphoid tissues from BN rats exposed to mercury (17,31–33).

After 2 weeks of mercury treatment, the percentage of RT6+ cells in lymph nodes was significantly decreased as compared to controls (100%, p = 0.0009), whereas spleen cells had a less marked change (55%, p = 0.0062). The total number of RT6.2+ lymphocytes of experimental BN rats was moderately increased in the spleen (78%, p = 0.0578), but was higher in lymph nodes (307%, p = 0.0033). On the other hand, the total number of RT6.2− cells was greatly increased and proportionally more than that of RT6.2+ cells (332%, p = 0.0084 in the spleen; 572%, p = 0.0062 in lymph nodes). As a consequence, the ratio of RT6+ to RT6− positive cells was higher both in lymph nodes and spleens of experimental BN rats (Fig. 2).

In the spleen, there was no significant change in the percentage of T-cells in experimental versus control rats (p = 0.2643), but the total numbers of T-cells increased by 173% after mercury treatment (p = 0.0045). Similarly, the percentage of spleen CD8+ lymphocytes did not show major changes (p = 0.3048), but their total number was increased (316%, p = 0.0313). In contrast, CD4+ cells were present in a lower percentage in experimental BN (38% change, p = 0.0004) but showed slightly higher numbers (119% change, p = 0.0114). The percentage of B-cells was not changed (p = 0.337), but their number increased by 230% (p = 0.002). Lymphocytes from cervical lymph nodes gave simi-

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**Figure 1.** Subcutaneous injection of rats with HgCl2 results in various degrees of splenomegaly and lymphadenopathy. Data obtained after 16 days of mercury treatment show a moderate increase of total cell numbers in the spleen (p = 0.0065) and lymph nodes (p = 0.0004) of LEW rats. Similarly, after 16 days of treatment with HgCl2, there was a considerable increase of total cell numbers in both BN (spleen, p = 0.0053; lymph nodes, p = 0.0001) and (BN X LEW) F1 hybrid rats (spleen, p = 0.01; lymph nodes, p = 0.0001).

**Figure 2.** Treatment with HgCl2 for 16 days causes an increase in the ratios of RT6+ to RT6− T-lymphocytes in both lymph nodes (LN) and spleen (SPL) of BN and (BN X LEW) F1 hybrids. Similarly treated LEW rats experience only minor variations of those ratios.

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on day 16 and 84% (p = 0.0189) on day 23. Numbers of CD4+ T-cells in both spleen and lymph nodes showed increases paralleling those of total cell numbers. Total numbers of B-cells increased in both spleen and lymph nodes, again paralleling the higher total cell numbers. Finally, numbers of lymphocytes positive for MHC class II antigens were increased on days 12, 16 and 23 (data not shown).

In summary, mercury treatment of LEW rats resulted in modest but significant increases in the number of spleen and lymph node cells, and as a consequence most subpopulations of lymphocytes were numerically increased. There were only slight and sporadic changes in the percentage of RT6+ T-cells, but their numbers were higher in experimental than in control animals. The ratio between RT6+ and
Table 3. Lymphocyte subpopulations in mercury-treated BN and (BN × LEW) F1 hybrids

| Strain       | Treatment | Cells | Total no. (× 10⁶) | %RT6.1 | %RT6.2 | %T-cells | %CD8 | %CD4 | %B-cells |
|--------------|-----------|-------|-------------------|--------|--------|----------|------|------|----------|
| BN           | HgCl₂     | Spleen| 535 ± 68          | 0      | 14.4 ± 1.3* | 32.6 ± 1.5 | 15.9 ± 1.6 | 28.5 ± 1.1 | 47.2 ± 1.4 |
|              | LN        |       | 484 ± 50          | 0      | 22.0 ± 2.9** | 34.6 ± 2.9 | 11.7 ± 2.0 | 32.4 ± 1.5 | 49.8 ± 3.4 |
| H₂O          | Spleen    |       | 166 ± 23          | 0      | 22.3 ± 2.4 | 35.9 ± 2.4 | 12.4 ± 2.1 | 39.3 ± 2.9 | 44.8 ± 1.6 |
|              | LN        |       | 50 ± 13           | 0      | 44.0 ± 5.9 | 64.0 ± 1.4 | 6.3 ± 0.4 | 54.4 ± 1.5 | 39.9 ± 1.6 |
| (BN × LEW) F₁| HgCl₂     | Spleen| 397 ± 33          | 13.1 ± 0.5*** | 17.2 ± 0.8*** | 38.2 ± 0.7 | 19.6 ± 0.6 | 31.0 ± 1.7 | 54.5 ± 1.1 |
|              | LN        |       | 580 ± 42          | 22.1 ± 1.4*** | 29.9 ± 1.6*** | 45.2 ± 1.4 | 17.5 ± 1.0 | 37.2 ± 1.3 | 51.0 ± 1.7 |
| H₂O          | Spleen    |       | 223 ± 30          | 27.5 ± 2.1 | 37.7 ± 2.0 | 47.4 ± 1.7 | 14.6 ± 0.8 | 40.1 ± 1.7 | 40.3 ± 1.1 |
|              | LN        |       | 41 ± 7            | 42.4 ± 1.0 | 60.3 ± 1.0 | 70.2 ± 2.3 | 10.7 ± 0.5 | 62.3 ± 1.7 | 20.1 ± 1.5 |

LN, lymph nodes. A total of 25 BN (18 experimental and 7 controls) and 32 F₁ hybrids (24 experimental and 8 controls) were sacrificed on day 16 from the start of the experiments. Flow cytometric data are expressed as means ± SEM.

Percentages of RT6.1⁺ and RT6.2⁺ T-lymphocytes significantly decreased in HgCl₂-treated rats as compared to H₂O-treated controls: *p = 0.0062; **p = 0.0003; ***p = 0.0001.

Lar patterns, with moderate decreases in the percentage of T-cells and CD4⁺ cells (89% and 68%, p = 0.0001 for both), and significant increases in their numbers (374% and 442%, p = 0.0001 for both). On the other hand, CD8⁻ cells presented no major percentage change (p = 0.1083) but had higher numbers (1625%, p = 0.0167). Finally, the percentage of B-cells in lymph nodes increased by 61% (p = 0.0028), and their number increased by 1417% (p = 0.0003) in experimental BN rats.

In summary, mercury-treated BN rats showed marked hyperplasia of peripheral lymphoid organs, significant decreases in the percentage of RT6⁺ cells, and proportionally higher numbers of RT6⁻ cells. As a result, the balance between these two subpopulations of lymphocytes was altered in favor of RT6⁻ cells.

Phenotypic Analysis of Lymphocyte Subpopulations in F₁ Hybrid Rats

Because F₁ hybrids between susceptible BN and resistant LEW rats are also susceptible to mercury-induced autoimmunity, we determined the phenotype of peripheral lymphocytes in experimental or control F₁ rats, with the following results.

As shown in Table 3 and Figure 1, the total number of spleen cells in experimental F₁ rats was increased by 78% as compared to controls (p = 0.01). A more striking increase in total cell numbers (1315%, p = 0.0001) occurred in cervical lymph nodes (Table 3).

The percentage of cells expressing the RT6 phenotypes was significantly decreased in both spleens and lymph nodes of experimental animals as compared to controls (Table 3). In the spleen, percentages of RT6.1⁺ and RT6.2⁺ were lower by 110% and 119% (p = 0.0001 for both). Similar reductions were observed in lymph nodes (92% and 102% change, p = 0.0001 for both). The total number of RT6.1⁻ and RT6.2⁻ spleen lymphocytes did not show significant changes, but RT6⁻ cells were increased (137%, p = 0.0016 for RT6.1 and 296%, p = 0.0001 for RT6.2). Therefore, the ratios of RT6⁺ to RT6⁻ spleen cells were increased by 197% for RT6.1 and 385% for RT6.2 (p = 0.0001 for both; Fig. 2). In cervical lymph nodes of experimental rats, the total number of RT6.1⁺ and RT6.2⁺ lymphocytes was significantly increased (623% and 587%, p = 0.0001 for both). However, RT6⁻ cells were present in even greater numbers (1114% change for RT6.1 and 2372% for RT6.2, p = 0.0001 for both), resulting in altered ratios of RT6⁻ to RT6⁺ cells (Fig. 2). An increase of 89% was observed for RT6.1/RT6.1⁺ (p = 0.0802) and 258% for RT6.2/RT6.2⁺ (p = 0.0064).

In the spleens of experimental F₁ animals, we observed a 24% decrease in the percentage of T-cells (p = 0.0001) and a 41% increase in T-cell numbers (p = 0.0766) as compared to controls. Both percentage and total numbers of spleen CD8⁻ lymphocytes were increased (35%, p = 0.0001 and 140%, p = 0.0021). In contrast, CD4⁻ cells were present in a lower percentage in the spleen of experimental F₁ rats (29% change, p = 0.0063), but their numbers were not significantly higher (p = 0.1156). Both percentage and number of spleen B-cells were increased (36%, p = 0.0001 and 142%, p = 0.0038). Lymphocytes from cervical lymph nodes gave similar patterns, with decreases in percentage of T- and CD4⁻ cells (55% and 68% change, p = 0.0001 in both cases) and striking increases in their numbers (816% and 732%, p = 0.0001 for both). On the other hand, lymph node CD8⁻ cells presented increases both in percentage and numbers (64%, p = 0.0004 and 2393%, p = 0.0001). Finally, there was a definite increase of B-lymphocytes in the lymph nodes of experimental F₁ rats: their percentages were 154% (p = 0.0001) higher, and their numbers rose by 3665% (p = 0.0001).

In summary, treatment of F₁ hybrids with mercury resulted in hyperplasia of peripheral lymphoid tissues similar to that observed in BN rats under the same circumstances. In addition, exposure to mercury was followed by significant decreases of RT6⁻ and increases of RT6⁺ cells. The resulting changes in the ratio between these lymphocyte subpopulations revealed that mercury had altered their balance. Thus, F₁ hybrids exhibited the same behavior of their mercury-susceptible parental strain (BN), both in their autoimmune responses to renal antigens and changes in lymphocyte subpopulations.

Discussion

The existence of T-suppressor cells is one of the most controversial topics of immunology. However, Todd and Steinman (34) state that “suppressor cells are back in fashion, since they refuse to go away and crop up repeatedly in autoimmune diseases as a plausible explanation for peripheral tolerance.” Previous studies relied exclusively on a few markers (e.g., CD4 and CD8) as representative of functional (helper/inducer versus suppressor/ cytotoxic, respectively) activities, which may explain both controversial results and lack of correlations between T-cell subsets, severity of autoimmune disease, and/or kinetics of autoimmune responses. The recent demonstration of T-cell heterogeneity, including various functional phenotypes of CD4⁻ cells (35–38), has led to more refined dissections of the complex regulatory network. In rats, peripheral T-cells differ in their expression of various membrane determinants, i.e., CD4, CD8, QCA-1, Thy-1, CD45 RC, and RT6. Thus, combining these markers allows the identification of a large number of phenotypically different subsets of peripheral T-lymphocytes (39). Previous investigations in our laboratory have demonstrated that BN rats repeatedly injected with merciruc chloride experience a relative decrease of peripheral RT6.2⁺ T-lymphocytes and an altered ratio of RT6⁻ to RT6⁻ T-cells (17). These changes coincide with the appearance of circulating autoantibodies to renal
Antigens (e.g., laminin). In the present study we extended our phenotypic analysis to rats of another strain (LEW) and examined additional numbers of BN rats as well as F1 hybrids between BN and LEW. We have confirmed that LEW and BN rats differ in their responses to the administration of HgCl2. It was known that LEW rats do not develop renal autoimmunity after mercury treatment (4). However, there was no information on the behavior of RT6+ T-cells after exposure of LEW rats to mercury, and it is of interest that their percentage did not significantly decrease. Numbers of RT6+ cells were actually higher in both spleens and lymph nodes of experimental rats, with increases that reached statistical significance. In the spleen, the ratio between RT6+ and RT6– T-lymphocytes showed a modest decrease only on day 30, whereas in the lymph nodes it was increased only on day 23, suggesting that treatment with mercury did not have consistent effects on this lymphocyte subpopulation. The lack of changes in this natural balance may explain the LEW resistance to the autoimmune effects of this metal. On the other hand, we confirmed our initial findings that BN rats injected with HgCl2 experience autoimmune responses to renal antigens as well as a decrease in the percentage of RT6+ cells. In spite of strikingly higher total numbers of spleen and lymph node cells, the numbers of RT6+ cells did not proportionally increase, resulting in an altered balance between RT6+ and RT6– T-lymphocytes (Fig. 2). This mercury-induced effect is quite similar to our previous results from three different groups of BN rats, which showed a definite decrease in the number of RT6+ spleen cells in one group and relative decreases in the other two (e.g., a 6% increase versus controls, in spite of a 52% increase in total cell numbers) (17). Differences in total numbers of RT6+ cells between various groups of BN rats seem to depend on the magnitude of mercury-induced splenomegaly and lymphadenopathy, which vary considerably with age, weight, and microbial status of the individual rats. However, percentage values of RT6+ cells and ratios between negative and positive cells are quite reproducible and provide a reliable indication of the immunotoxic effects of mercury on this subpopulation of BN rat lymphocytes.

We observed that F1 hybrid rats (i.e., hybrids between susceptible and resistant rats), behaved like their BN parent strain when exposed to mercury: they had both renal autoimmunity and significantly lower percentages of RT6+ T-lymphocytes. As far as autoimmunity to kidney antigens is concerned, there was no detectable difference between BN and F1 rats (Table 1). All animals had linear immunoglobulin deposits in their kidneys; previous work has demonstrated that such deposits contain antibodies to laminin and other renal antigens (14, 15). Antibodies to laminin and rat GBM were also present in the sera of most experimental rats. Flow cytometric analysis showed that both RT6.1 and RT6.2 surface markers are expressed on lymphocytes of F1 rats. Control animals had a higher percentage of RT6.2+ cells than BN rats and a percentage of RT6.1+ cells similar to that of LEW (Tables 2 and 3). Previous studies by single-label immunofluorescence staining of lymph node cells obtained from F1 hybrids between Wistar Furth (RT6.2+) and diabetes-prone or diabetes-resistant BB (RT6.1+) rats also revealed the presence of RT6.1+ and RT6.2+ lymphocytes in both hybrids (40). Dual-label flow cytometric analysis of these lymphocytes for RT6.1 and RT6.2 allo-antigens has shown that the two molecules are co-expressed on most of the cells. In addition, those F1 hybrids had a higher percentage of RT6.2+ than RT6.1+ cells, which is confirmed by our findings in hybrids between two additional rat strains. The reasons for such a difference are unknown but are probably related to the epitope(s) recognized by the monoclonal antibodies against RT6 alloantigens rather than lack of expression of RT6.1 on some RT6.2+ cells. In any case, experimental F1 rats had a lower percentage of both RT6.1+ and RT6.2+ cells in their spleens and lymph nodes. Again, total numbers of these cells were decreased in the spleen. On the other hand, as previously observed in BN rats, there was a relative decrease of RT6+ cells in cervical lymph nodes, i.e., their number did not show the striking increase observed in total cell numbers. As a consequence, these changes resulted in an altered balance within the RT6 T-lymphocyte population, as demonstrated by the ratios between positive and negative cells (Fig. 2).

Exposure to mercury had other immunotoxic effects on peripheral lymphoid tissues (see Fig. 1). Total cell numbers increased in the spleen and lymph nodes of all experimental rats; however, the increase was rather modest in LEW rats as compared to that observed in BN rats. Changes in the spleens of F1 hybrids were intermediate between those of the parental strains, whereas numbers in lymph nodes were much higher. Other investigators have previously reported that spleens and lymph nodes were not much enlarged in LEW rats injected with HgCl2, and there were no major changes in total cell numbers (18, 33, 41). However, the modest increases we observed may be easily explained by differences in age and weight of our LEW rats or the use of a different substrate, possibly more susceptible to mercury effects. The marked proliferation of BN spleen and lymph node cells after treatment with HgCl2 has been previously noted by various investigators (17, 18, 32, 33, 41). On the other hand, the lymphadenopathy and splenomegaly of F1 hybrids have not received much attention.

In addition to numerical increases of spleen and lymph node cells, we also noticed changes in most lymphocyte subpopulations. For example, flow cytometry of lymph nodes from experimental LEW rats revealed a percentage decrease of B-cells on day 12 and an increase on days 16 and 23. Parallel percentage changes of IA+ cells were also observed on the same days. The percentage of T-cells in the same lymph nodes never varied significantly and that of CD8+ cells was increased only on day 23. In contrast, CD4+ cells differed on each day of treatment, with a percentage increase on day 12 and decreases thereafter. Values for the spleen were more constant, with the only significant percentage changes noted on day 30, when there was a decrease of B- and IA+ cells. On the other hand, total cell numbers of almost all subsets were higher on days 12, 16, and 23 of treatment, proportional to the moderate increase in total cell numbers. Data for experimental BN rats were obtained only on day 16, i.e., at a time when their autoimmune responses to renal antigens are at their peak (17). B-cell percentages and numbers were both higher than in controls, whereas T-cell percentages decreased and numbers increased. Percentages and numbers of CD8+ T-lymphocytes were increased. In contrast, percentages of CD4+ T-cells decreased and their numbers increased. Almost identical changes were observed in spleen and lymph nodes obtained from F1 hybrids on day 16 of treatment with HgCl2.

Of particular interest is our finding that both percentage and total numbers of CD8+ T-lymphocytes did not show consistent and significant increases in the spleens of experimental LEW rats. In the lymph nodes, CD8+ cells increased in percentage only on day 23 and in total numbers only on days 16 and 23, proportional to the higher total cell numbers. In contrast, experimental BN and F1 rats had increased percentages as well as higher numbers of these lymphocytes. These data show that BN and F1 rats, at the peak of autoimmune responses induced by the administration of mercury, have increased levels (both as a percentage and numerically) of CD8+ T-lymphocytes, whereas LEW rats, resistant to the autoimmune effects of this metal, usually show only numerical increases. On this basis, it is difficult to
attribute an immunosuppressive role to CD8+ T-lymphocytes either in LEW or BN rats. In earlier studies, Pelletier et al. (18,20) observed significant increases in both percentage and/or numbers of CD8+ T-cells in LEW rats on days 14, 28, and 35 of mercury treatment. The same group reported that the administration of HgCl2 to LEW rats for 2 weeks before immunization with certain autoantigens had immunosuppressive effects on the experimental induction of autoimmunity (e.g., Heymann’s nephritis and EAE) (19,42). As far as BN rats are concerned, sequential studies of peripheral blood lymphocyte populations during HgCl2 treatment have shown a transient decrease in the percentage of CD8+ T-cells before the appearance of serum anti-GBM antibodies, followed by elevated levels of these cells during down-regulation of the response (43). Aten et al. (32) also noted that CD8 levels were higher in BN rats on day 21 than on day 0. These observations led to the suggestion that CD8+ cells, which at that time were still defined as “suppressor/cytotoxic” T-lymphocytes, might have an immunoregulatory role in both LEW and BN rats. Differences in techniques of phenotypic analysis and the use of various substrains of rats may explain the discrepancies between the data available in the literature. In any case, considerable doubts have later been raised about the existence of T-suppressor cells in general and the immunosuppressive properties of CD8+ T-lymphocytes in particular. Our findings suggest that increases of CD8+ T-cells may actually have no major role in the lack of autoimmune responses or the down-regulation of renal autoimmunity observed in LEW and BN rats, respectively, after mercury treatment.

Finally, our data underline the importance of a complete and detailed phenotypic analysis of lymphoid cells in studies of immunotoxicity and xenobiotic-induced autoimmunity. Traditional markers such as CD4 and CD8 may not be sufficient to characterize different subpopulations of T-cells, in view of the heterogeneity demonstrated by functional studies (38). The use of additional surface markers (e.g., the RT6 alloantigens) together with cytokine profiles of T-cell clones may be necessary for a better understanding of immunoregulatory processes. T-cells of the RT6 phenotype are known to be severely decreased in spontaneously hyperglycemic diabetes-prone BB/Wor rats, and their deficiency may be an important predisposing factor in susceptibility to insulin-dependent diabetes mellitus. It has also been suggested that RT6 T-cells have a regulatory role in self-tolerance (44). The parallel changes of RT6+ T-lymphocytes observed in both BN and F1 hybrids as well as the opposite behavior detected in LEW rats also suggest a regulatory role of RT6 lymphocytes.

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