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$HgCl_2$ induces T and B cells to proliferate and differentiate in BN rats

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SUMMARY

Mercuric chloride induces in Brown-Norway (BN) rats an autoimmune disease characterized by the production of various autoantibodies and by a marked increase in the IgE serum concentration. This agent is responsible for a T dependent polyclonal activation of B cells, which is probably due to the emergence of autoreactive T cells. The aim of this study was to evaluate the effect of $HgCl_2$ injections on lymphoid organs and on the serum concentration of the various Ig isotypes. $HgCl_2$ induced (1) a lymphoproliferation in spleen and lymph nodes involving B and T helper cells while the number of T suppressor/cytotoxic cells was not modified, (2) an increase in the number of Ig containing cells resulting in a rise in all serum Ig isotypes, and (3) an early thymic atrophy probably immunologically mediated, which was not involved in the induction phase of the disease since adult thymectomy had no effect. These findings demonstrate that the polyclonal effect of $HgCl_2$ is not isotype-restricted although the IgE response is predominantly affected and they support evidence for a major role for an excess of T help in the $HgCl_2$-induced polyclonal activation of B cells. It was also observed that B cell areas are present in normal BN rat thymuses, the potential role of which in the induction of autoimmunity remains to be investigated.

Keywords rat mercuric chloride polyclonal activation lymphoid organs IgE

INTRODUCTION

Mercuric chloride has been shown to induce an autoimmune disease in Brown-Norway (BN) rats characterized by the production of various antibodies to self and non-self antigens, and by a tremendous increase in total serum IgE concentration (Prouvost-Danon *et al.*, 1981; Pelletier *et al.*, 1987a). A biphasic autoimmune glomerulonephritis occurs: initially anti-glomerular basement membrane (GBM) antibodies are produced resulting in linear IgG deposition along glomerular capillary walls from day 15; after the second week, granular IgG deposits appear which are responsible for an immune complex type glomerulonephritis (Pelletier *et al.*, 1987a). Other studies led to the conclusion that $HgCl_2$ induces a T cell dependent polyclonal activation of B cells in BN rats (Hirsch *et al.*, 1982). More recently, it was demonstrated that $HgCl_2$-injected BN rats developed autoreactive T cells that could be responsible for the polyclonal activation of B cells (Pelletier *et al.*, 1986).

The aim of the present study was to evaluate sequentially the effect of $HgCl_2$ on the distribution of lymphocyte subpopulations in lymphoid organs, and to determine whether or not this agent is responsible for isotype-restricted increase in serum Ig concentration. It will be shown that the number of B cells and T helper cells increases in $HgCl_2$-injected BN rats while the number of suppressor/cytotoxic T cells is not modified. The effect on B cells is not isotype-restricted although total serum IgE level is preferentially affected. This study shows that $HgCl_2$ is responsible for an activation of T helper cells and B lymphocytes leading to polyclonal B cell differentiation.

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MATERIALS AND METHODS

Rats

Eight-to-twelve-week old male and female BN rats were used throughout this study. They were initially obtained from the CSEAL (Orléans—La Source, France) and were then maintained by brother–sister mating in our own animal house.

Monoclonal antibodies and antisera

Mouse monoclonal antibodies (MoAb) specific for rat lymphocytes were purchased from Seralab (Crawley Down, Sussex, UK). The W3/13 MoAb is specific for T cells (Brown *et al.*, 1981), W3/25 for helper/inducer T cells (Barclay, 1981), OX8 for suppressor/cytotoxic T cells (Brideau *et al.*, 1980) and OX6 for a polymorphic rat Ia determinant (McMaster & Williams, 1979). Fluoresceinated rabbit $F(ab')_2$ anti-mouse Ig and $F(ab')_2$ anti-rat Ig were supplied by Zymed (San Francisco, CA) and by Cappel Laboratories (Cochranville, PA) respectively, Goat

anti-rat IgG2a, IgG2b and IgA, as well as rabbit anti-rat IgG1, IgG2c and IgM antisera, were obtained as previously described (Bazin, Beckers & Querinjean, 1974) and rendered heavy-chain-specific by absorption on monoclonal rat kappa light chains (Bazin *et al.*, 1972). These antisera were precipitated at 40% ammonium sulphate saturation and fluoresceinated (Sapin, Druet & Druet, 1977). Mouse MoAb anti-rat kappa chain MARK-1 (Bazin *et al.*, 1984) was peroxidase-labelled and used to identify B lymphocytes (95% of murine B lymphocytes express kappa light chains) in immunomorphological studies. Sheep IgG anti-rat Ig were obtained as described by Sapin *et al.* (1977) and rendered specific for rat Fc fragments by absorption on rat $F(ab')_2$ fragments (Binaghi, Oriol & Boussac-Aron, 1967) coupled to Sepharose beads. These antibodies were then peroxidase-labelled. Rabbit anti-fibronectin and anti-cytokeratin antisera were purchased from Dakopatts (Glostrup, Denmark) and the Pasteur Institute (Lyon, France) respectively. Peroxidase-labelled rabbit IgG anti-mouse Ig were purchased from Dakopatts, goat anti-rabbit Ig antiserum from Nordic (Tilburg, The Netherlands), and peroxidase anti-peroxidase (PAP) reagents from Biolyon (Lyon, France).

Experimental procedure

Mercuric chloride was injected subcutaneously three times a week at a dose of 100 μg per 100 g body weight (Sapin *et al.*, 1977). Control rats received the same volume of H_2O adjusted to the same pH (3·8) as the mercury solution.

Groups of rats tested

Four groups of rats were tested. In the first group, $HgCl_2$-injected and control BN rats (10 rats per group) were sequentially bled for determination of serum Ig level on days 0, 7, 14, 21 and 28 and killed on day 42. In the second group $HgCl_2$-injected and control BN rats were killed on days 7, 14, 21 and 42 (six to eight rats per day and per group) for phenotypic characterization of spleen, lymph node and thymus cell populations. The third group received cyclophosphamide (20 mg/kg every other day, intraperitoneally) from day 0 in addition to $HgCl_2$; these rats were killed on days 7, 14, 21, 28 and 42 (five rats per day). In the fourth group, 10 rats were thymectomized and injected with $HgCl_2$ 1 month later (five rats) or with control solution (five rats). Ten other rats were sham-thymectomized and injected with $HgCl_2$ (five rats) or with control solution (five rats). These rats were sequentially bled as in the first group. An open wedge renal biopsy was performed on day 15 after the first injection of $HgCl_2$ and kidneys were examined by direct immunofluorescence with a fluoresceinated anti-rat Ig conjugate on days 15 and 42 to detect linear and/or granular IgG.

Determination of serum Ig concentrations

Total serum IgE level was determined using an ELISA described by Sapin *et al.* (1984). The following ELISA were designed in order to determine serum concentrations of the other Ig classes and subclasses. Ninety-six-well round-bottomed microtitre plates (Nunc, Kamstrup, Denmark) were coated with 100 μl of the various ammonium sulphate precipitated antisera. Anti-IgG2a, IgG2b, and IgA reagents were used at 2 μg/ml and anti-IgG1, IgG2c and IgM at 0·5 μg/ml in phosphate buffered saline (PBS). After 16 h at 4°C, the plates were washed twice with 0·9% NaCl containing 0·05% Tween 20 (NaCl-Tween). Wells were then filled with 100 μl of sera to be tested at three dilutions (1:1000, 1:5000, 1:10,000). Each dilution was tested in duplicate. Plates were incubated for 45 min and washed with NaCl-Tween. Wells were filled with peroxidase-labelled sheep anti-rat Ig conjugate. Plates were again incubated for 90 min at 37°C, then washed and tested for peroxidase activity as described elsewhere (Sapin *et al.*, 1984). For each assay, a reference curve was built using a normal rat serum sample containing known amounts of the Ig classes and subclasses (Bazin *et al.*, 1974). The interplate and intraplate variability was at most $15 \pm 5\%$ and $9 \pm 4\%$ respectively.

Preparation of cell suspensions and determination of cell phenotype

Spleen, lymph nodes and thymus were removed on days 7, 14, 21, and 42. Cell suspensions were prepared and stained with $F(ab')_2$ anti-rat Ig for B cells, W3/13, W3/25, and OX8 MoAb for T cells, and OX6 MoAb for Ia^+ cells according to Pelletier *et al.* (1986).

Characterization of Ig containing cells

In selected experiments, the number of Ig-containing cells was determined in the spleen of control rats and of rats injected with $HgCl_2$ for 14 days. Cells were stained according to Katona *et al.* (1983) with fluoresceinated anti-IgG, IgA and IgE conjugates at the appropriate dilution. The number of IgG containing cells was assessed by using a mixture of the four fluoresceinated anti-rat IgG subclass reagents.

Immunohistochemistry

Spleen, latero-aortic lymph nodes, and thymus from killed rats were immediately snap-frozen in liquid nitrogen. Cryostat sections were cut at 6 μm, allowed to dry overnight at 4°C, fixed for 10 min in acetone at room temperature, and air-dried before immunostaining. Sections from spleen, lymph node and thymus samples were sequentially reacted with MoAb, peroxidase-conjugated rabbit IgG anti-mouse Ig, peroxidase-conjugated goat anti-rabbit Ig antiserum and diaminobenzidine-H_2O_2 as a substrate. Peroxidase-conjugated rabbit IgG anti-mouse Ig was previously depleted of any rat Ig cross-reactivity by absorption on normal rat serum. Anti-kappa light chain MoAb was revealed by peroxidase-conjugated rabbit IgG anti-mouse Ig antibodies. Anti-fibronectin and anti-cytokeratin antibodies were revealed by the PAP technique. All the slides were counterstained with Mayer's haematoxylin. Controls were obtained by omission of primary antibodies.

Statistical analysis

Variability of serum Ig class and subclass concentration was tested within each group on the various days using the *F*-test. Moreover, daily values in $HgCl_2$-injected BN rats were compared to those of control rats using Student's *t*-test. A similar approach was used to compare the number of cells and cell subsets in lymphoid organs.

RESULTS

Serum Ig concentrations

As shown in Fig. 1, a significant increase in serum IgM, IgG1, IgG2b, IgG2c and IgA concentrations was observed in rats injected with $HgCl_2$ when compared to day 0 values of control rats on the same days. The rise in serum IgE concentration

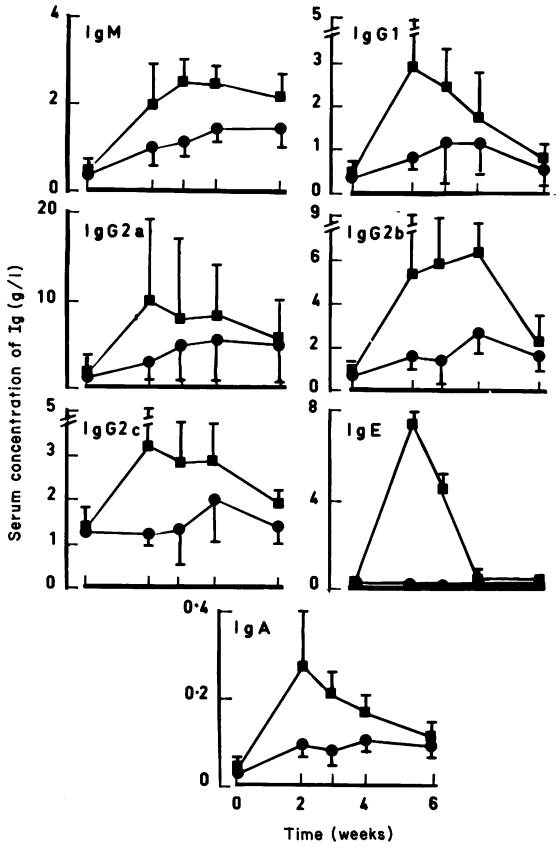

Fig. 1. Sequential determination of serum Ig isotypes in $HgCl_2$-injected (■) and control (●) BN rats. All the isotypes except IgG 2a were significantly increased on days 14 and 21 in $HgCl_2$-injected BN rats ($P<0.01$) and singularly the IgE ($P<0.001$). The IgE level in $HgCl_2$-injected BN rats remained higher than in control ($P<0.01$) on days 28 and 42.

described by Prouvost-Danon *et al.* (1981) was much more impressive. For all these classes or subclasses, a peak was reached between the second and third weeks. Values for all immunoglobulins except IgE then progressively declined to levels similar to those of control rats by day 42. Serum IgE concentration, although much lower than on day 14, remained significantly higher when compared to control rats. Serum IgG2a concentration, although increased in rats injected with $HgCl_2$, did not differ significantly at any time from that of control rats. A significant increase in serum IgM, IgG2a and IgA concentrations was observed in control rats at the time of death when compared to day 0 values. Such a progressive increase has been previously described in young normal rats (MacGhee, Michalek & Ghanta, 1975).

Determination of cell phenotype

As shown in Fig. 2, the number of total spleen cells, B, T, T helper and Ia^+ cells, was significantly but transiently increased on days 7 and 14 in rats injected with $HgCl_2$ as compared to control rats. The percentage of B cells was increased in $HgCl_2$-injected rats when compared to control rats on days 7 (48 ± 4 vs $39 \pm 5\%$) and 14 (60 ± 5 vs $42 \pm 5\%$) while the percentage of T helper cells was not different. Findings in mesenteric lymph nodes (not shown) were similar to those described in the spleen.

Thymic tissue was also examined in these animals. From day 6, the thymus of $HgCl_2$-injected rats was surrounded by multiple nodular formations that were shown to be lymph nodes by histological examination. These formations were carefully removed and the number of cells was assessed in the remaining tissue. As shown in Fig. 3, the number of thymocytes decreased rapidly after day 7 and was reduced by more than 90% from the second week. However the percentage of W3/13, W3/25 and OX8 positive cells in the remaining tissue did not differ from that of control rats while there was a slight but significant increase in the percentage of B cells ($5{\cdot}5 \pm 1{\cdot}5\%$ versus $1 \pm 0{\cdot}5\%$; $P<0.01$).

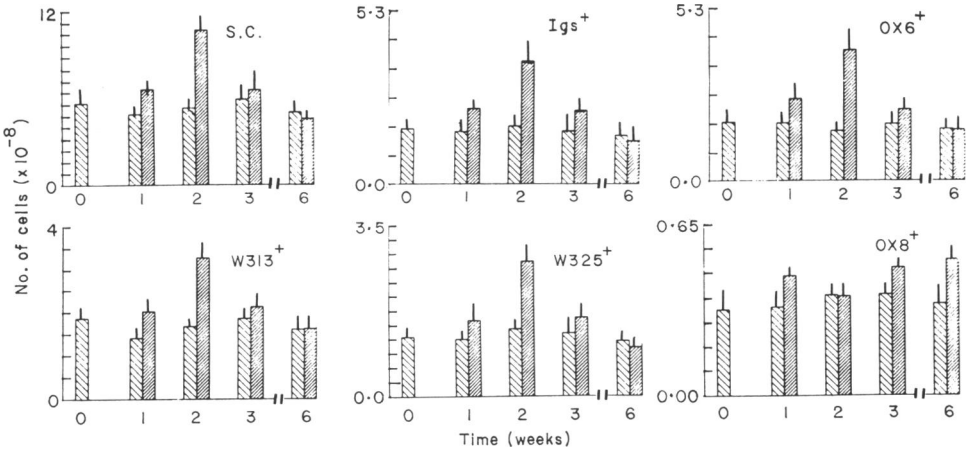

Fig. 2. Sequential determination of the total number of spleen cells (SC) and of the number of B (IgS^+), Ia ($OX6^+$), T ($W3/13^+$), T helper/inducer ($W3/25^+$) and T suppressor/cytotoxic ($OX8^+$) cells in $HgCl_2$-injected (■) and control (▨) BN rats. On day 14 the number of B cells, Ia^+ cells, T and T $W3/25^+$ cells increased significantly in $HgCl_2$-injected BN rats when compared to control ($P<0.005$, $P<0.005$, $P<0.01$, $P<0.01$, respectively).

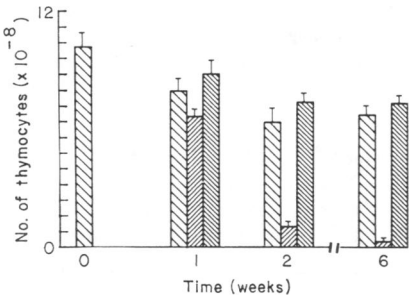

Fig. 3. Sequential determination of the number of thymocytes in $HgCl_2$-injected (■), control (▨), and cyclophosphamide-treated, $HgCl_2$-injected BN rats (■). The number of thymocytes decreased significantly from day 14 in $HgCl_2$-injected BN rats ($P < 0{\cdot}001$).

In rats treated with cyclophosphamide, the number and phenotype of thymus cells remained unchanged throughout the study (Fig. 3) As described by Pelletier *et al.* (1987b), the other autoimmune abnormalities were also prevented.

Immunomorphological study of lymphoid organs

Control BN rats. The immunohistological study of lymph nodes, spleen and thymus gave results similar to those previously reported by Barclay (1981) and Mason *et al.* (1983). Unexpectedly however, lymphoid nodules of B cells (kappa chain$^+$, OX6$^+$, W3/13$^-$, W3/25$^-$, OX8$^-$) were observed at the corticomedullary junction within the thymus parenchyma (Fig. 4a). They were outlined by keratin-positive epithelial cells (Fig. 4b). Vascular structures, stained with the anti-fibronectin antibodies were found around, but not within, these follicles

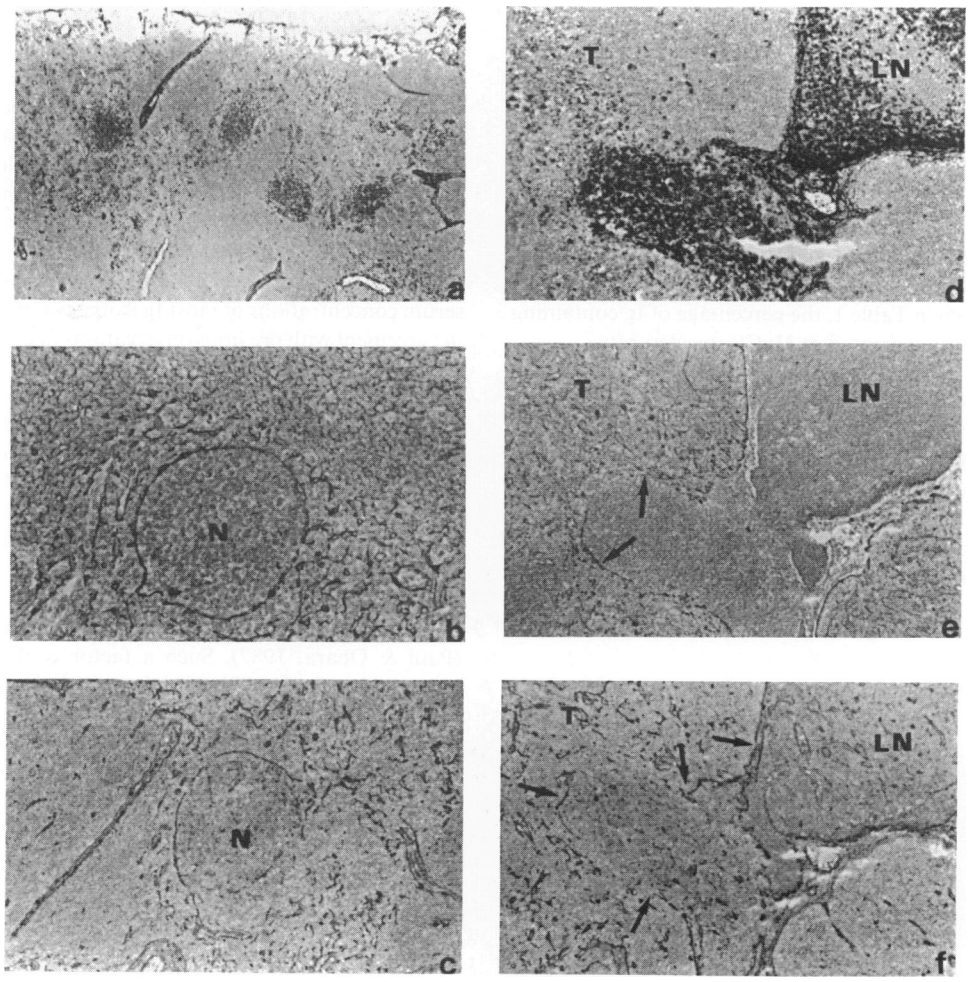

Fig. 4. Thumus cryostat sections from a control BN rat. (a) Staining with the anti-rat kappa light chain MoAb (× 20); four nodules are labelled at the corticomedullary junction. These nodules (N) are devoid of keratin-positive epithelial cells; keratin-positive cells are observed at their periphery and in the medulla (b) (× 200). Fibronectin-positive vascular structures are seen dispersed in the medulla but not in the nodule (c) (× 200). Serial sections (d, e, f) of an expansion of a B cell area from a parathymic lymph node (LN) penetrating the thymus (T) (× 100). Staining with the anti-rat kappa light chain MoAb (d), anti-keratin and anti-fibronectin antibodies (e and f). Lymph node invagination (arrow) compresses thymic keratin positive cells (e); thymic capsule (arrow) stained with anti-fibronectin antibody (f) is interrupted at the point of contact with the B cell area.

(Fig. 4c). These follicles could represent lymph node enclaves, since in some sections expansions of B cell areas from parathymic lymph nodes were seen to pentrate the thymus (Fig. 4e) without fibrous tissue interposition between thymus and lymph nodes (Fig. 4f) as shown by anti-fibronectin staining.

$HgCl_2$-injected BN rats

Spleen and lymph nodes were examined on days 5–7 (four rats), 14–17 (seven rats), and 30–41 (three rats). The thymus was examined in only three, six and two of these rats respectively.

From day 5, an impressive hypertrophy of parathymic lymph nodes was observed in all the rats until day 41. In three rats, no thymus tissue was found. In the eight other rats, thymus structures were compressed by the hyperplastic lymph nodes and, in some sections, no fibrous capsule could be observed between thymus and lymph nodes. The remaining thymic structures contained the same distribution of lymphocyte populations as in control rats.

In the lymph nodes a follicular hyperplasia was observed in 11 out of the 14 rats tested. It was marked in eight of them, including the four rats examined on days 5–7, and moderate in the remaining three other rats. In all the rats, medullary cords were considerably thicker with a high cellular density resulting in a homogenous pattern in 12 out of 14 rats. No clearcut changes were noted in the paracortex. Follicular hyperplasia was also observed in the spleens of all but two rats.

Immunoglobulin containing cells

The percentage of IgG, IgA and IgE containing cells was determined in four control rats and in four $HgCl_2$-injected rats on day 14. As shown in Table 1, the percentage of Ig containing cells was significantly increased in $HgCl_2$-injected rats.

Effect of thymectomy

The disease observed (degree and time course of both proteinuria and serum IgE concentration, presence of kidney bound anti-GBM abs on day 15 and of granular deposits on day 42) was similar whether $HgCl_2$-injected BN rats were thymectomized or not. No abnormalities were observed in thymectomized rats who did not receive $HgCl_2$.

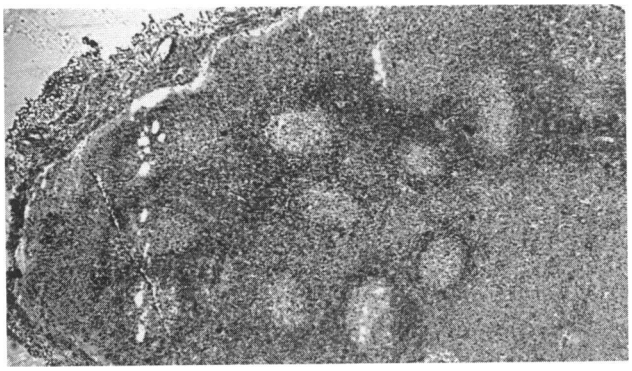

Fig. 5. Lymph node cryostat sections from a BN rat injected with $HgCl_2$ for 14 days (× 100). Staining with W3/25 MoAb shows the unlabelled follicular hyperplasia with well-developed germinal centres.

Table 1. Percentage of Ig containing cells in the spleen of $HgCl_2$-injected BN rats at day 14

Rats		Percent of stained cells		
(No)	$HgCl_2$	IgG	IgA	IgE
BN (4)	–	<0·1	<0·1	<0·1
BN (4)	+	0·8±0·4*	0·5±0·005*	1·5±1·2*

* $P<0·005$ when compared to values from control BN rats.

DISCUSSION

Data presented show that $HgCl_2$ is responsible for an increase in the number of B and T helper cells in BN rats and for a follicular hyperplasia. The increased number of $W3/13^+$ cells in the medulla, partly identified as immunoblasts, could contribute to the increment in $W3/13^+$ cells observed by membrane immunofluorescence. These findings agree with our previous studies showing that $HgCl_2$ is responsible for a T dependent polyclonal activation of B cells (Hirsch *et al.*, 1982) probably related to the emergence of autoreactive T cells in this model (Pelletier *et al.*, 1986). The present study also shows that the number of surface Ig^+ as well as that of IgG, IgA and IgE-containing cells was increased. This demonstrates that B cells are polyclonally induced to proliferate and differentiate. As a consequence, serum concentrations of most Ig isotypes were increased. This is in agreement with previous observations in BN rats (Pusey *et al.*, 1983; Andres, 1984) and mice (Hultman & Enestrom, 1987) given $HgCl_2$. The kinetics of the increase in the serum level of the various Ig isotypes was similar to that observed for IgE. This suggests similar spontaneous autoregulation which could involve suppressor T cells (Bowman *et al.*, 1984) and or auto-anti-idiotypic antibodies (Chalopin & Lockwood, 1984).

It is noteworthy that IgE B cells were preferentially affected leading to the striking increase in total serum IgE level (Prouvost-Danon *et al.*, 1981). Interleukin 4, produced by mouse activated T cells, promotes preferential IgE production (Paul & Ohara, 1987). Such a factor could be produced by autoreactive T cells of $HgCl_2$-injected autoimmune rats. It could also explain the selective enhancement of IgE production by normal human blood mononuclear cells cultured in the presence of pokeweed mitogen and $HgCl_2$ (Kimata, Shinomiya & Mikawa, 1983). The IgE response is also under suppressor T cell control (Geha *et al.*, 1981); although the number of $OX8^+$ (suppressor/cytotoxic) T cells in $HgCl_2$-injected BN rats did not differ from that of control rats, a defect in the function of T suppressor cells cannot be ruled out. Studies are in progress to clarify these points.

Botazzo *et al.* (1986) have shown the role of aberrant epithelial expression of class II molecules in the induction of various organ-specific autoimmune diseases. In the mercury model, the number of Ia^+ cells did not exceed that of Ig^+ cells, and immunohistological analysis of kidney (not shown), and of lymphoid organs did not reveal Ia expression in unexpected locations. However, a chemically-induced modification of Ia molecules, undetected by the OX6 MoAb, cannot be ruled out

and an increase in the number of Ia molecules per cell has not been looked for.

This study also provides interesting data concerning the thymus. In normal BN rats, a greater number of B cells than previously reported (Mason *et al.*, 1983) has been found and nodules of B cells were observed at the cortico-medullary junction. Immunohistological analysis strongly suggested that these nodules corresponded to lymph node invaginations within the thymus. This intriguing finding has not been previously reported in normal rats. Lymphoid follicles containing B cells have been observed in old (NZBxSJL) F1 autoimmune mice (Dumont & Robert, 1980). The physiological meaning of such B cell areas and their potential role in the pathogenesis of autoimmune disorders remain to be investigated. Also of interest was the finding of a thymic atrophy in diseased rats, in agreement with a report by Aten *et al.* (1986). This was not a consequence of a direct toxic effect of $HgCl_2$ since atrophy was not observed in cyclophosphamide-treated and $HgCl_2$-injected BN rats (Pelletier *et al.*, 1987b). Thymic atrophy is more likely a consequence of autoimmunity but its mechanism remains to be elucidated. Similar observations have been made in lupus-prone mice (Andrews *et al.*, 1978). Thymic atrophy is unlikely to play a role at least in the induction phase of the disease since no differences were observed following adult thymectomy.

This study shows that $HgCl_2$ induces in BN rats a proliferation of T helper cells and of B cells that is not isotype-restricted as well as a thymic atrophy. The disease observed shares many features with lupus syndromes and may be considered as a model of a chemical-induced lupus.

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