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Mercuric Chloride-Induced Autoimmunity in the Brown Norway Rat

Cellular Kinetics and Major Histocompatibility Complex Antigen Expression

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$HgCl_2$ induces an autoimmune syndrome in Brown Norway rats that involves synthesis of anti-glomerular basement membrane (GBM) antibodies and development of nephritis with high proteinuria. $HgCl_2$-induced changes in the composition of leukocyte populations and in the expression of MHC antigens in lymphoid and nonlymphoid organs were investigated by flow cytometry and immunohistochemistry. An early increase of CD4+ splenocytes was followed by a transient proliferation of CD4+ as well as CD8+ and B lymphocytes in peripheral lymphoid organs; in contrast, progressive depletion of the thymic cortex was found. B lymphocyte activation involved mainly the IgG1 and IgE isotypes. Nonlymphoid organs were infiltrated by MHC class II antigen expressing CD4+ and CD8+ T lymphocytes and monocytes; secondary to infiltration, mainly epithelial cells, being the main target of infiltrating cells, showed increased expression of MHC antigens. In glomeruli a 2.7-fold increase of CD8+ lymphocytes occurred after $HgCl_2$-administration. The diverse autoimmune phenomena observed in this study fit with the hypothesized involvement of T lymphocytes autoreactive with MHC class II antigens. Apart from anti-GBM autoantibodies, a role for autoreactive CD8+ T lymphocytes must be considered in the pathogenesis of the $HgCl_2$-induced autoimmune syndrome. (Am J Pathol 1988, 133:127–138)

USING LOW DOSES of mercuric chloride, a genetically restricted autoimmune syndrome can be induced in Brown Norway rats that involves polyclonal B lymphocyte activation, hypergammaglobulinemia, high IgE serum levels, synthesis of anti-nuclear and anti-GBM antibodies and development of proteinuria.[1–4] Evidence that the induction of the syndrome is T lymphocyte-dependent was obtained by prevention of autoimmune phenomena with cyclosporin A treatment during administration of mercuric chloride,[5,6] and by T lymphocyte depletion and reconstitution experiments.[7] Recently, the proliferation of autoreactive T lymphocytes was demonstrated to be stimulated *in vitro* by $HgCl_2$-pretreated CD4+ T lymphocytes in the presence of normal MHC class II antigen-bearing cells.[8] The nature of these MHC class II antigen positive cells has not yet been established.

The pathogenesis of the proteinuria in this model is at present unknown. Both humoral and cellular mechanisms may play a role in its induction. Autoantibody synthesis against several GBM components has been reported.[9] Complement activation is not necessary for the development of proteinuria in this model.[10] Ultrastructural studies of glomeruli revealed an influx of polymorphonuclear leukocytes and monocytes during the proteinuric phase after $HgCl_2$ administration; no increase in the number of lymphocytes was observed.[11] Besides the kidney, other organs may be involved as a target in the $HgCl_2$-induced au-

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Table 1—Monoclonal Antibodies

MAb	Isotype	Specificity	Reference
MRC OX6*	IgG1	MHC class II antigens (RT1.B)	16
1F119†	IgM	MHC class II antigens (RT1.B); preference for dendritic cells	17
MRC OX17	IgG1	MHC class II antigens (RT1.D)	18
ER13	IgG1	MHC class II antigens (RT1.D)	19
MRC OX18	IgG1	MHC class I antigens (RT1.A)	18
MRC OX19	IgG1	T lymphocytes	20
MRC OX8	IgG1	CD8; cytotoxic and suppressor T lymphocytes; NK cells	21, 22
ER2	IgG1	CD4; helper and inducer T lymphocytes; macrophages; (W3/25-like)	23
HIS17‡	IgG1	all T lymphocytes; plasmacells; (W3/13-like)	24
HIS14	IgG2b	B lymphocytes	25
HIS22	IgM	Coronatype B lymphocytes	25
ED1§	IgG1	Monocytes and macrophages	26
ED2	IgG2a	Tissue macrophages	26

* MRC OX hybridomas were a gift of Dr. A. F. Williams (University of Oxford, UK).

† A gift of Dr. L. Nagelkerken (TNO-IVEG, Rijswijk, The Netherlands).

‡ HIS MAb's were generously provided by Dr. F. G. M. Kroese (University of Groningen, The Netherlands).

§ ED MAbs were a gift of Dr. C. D. Dijkstra (Free University, Amsterdam, The Netherlands).

toimmune syndrome, as shown by extrarenal deposition of immune complexes.[12] No information is available concerning cellular immune mechanisms in these organs.

The response of CD4+ lymphocytes to antigen is restricted by self MHC class II antigens. Tolerance to autoantigens may be maintained in part by the absence of MHC class II antigens on most nonlymphoid tissues, thereby preventing the possible activation of CD4+ clones specific for autoantigens. Induction of MHC class II antigen expression on nonlymphoid tissue may cause effective presentation of self antigens, resulting in autoimmune reactivity.[13,14]

The composition of lymphocyte populations in peripheral lymphoid organs and the phenotype of leukocytes in the kidney and other nonlymphoid organs in various stages of the syndrome were studied by flow-cytometry and immunohistochemistry. Special attention was given to changes in the expression of MHC antigens.

Materials and Methods

Animals

Inbred 3–4-month-old male Brown Norway (BN) rats, weighing 250–335 g were used. The BN line was originally obtained from the CSEAL (Orléans, La Source, France) and maintained under conventional conditions. The animals were fed RMH-B chow (Hope Farms, Woerden, The Netherlands) and received tap water *ad libitum*.

Experimental Protocol

BN rats (n = 26) were given $HgCl_2$ (Sigma Chemical Co., St. Louis, MO) subcutaneously at a dose of 1 mg/kg body weight at days 1, 3, 5, 7, and 9. Control animals (n = 10) did not receive $HgCl_2$.

Proteinuria was measured by the biuret method at day 0 and thereafter at least twice a week. Rats were maintained in metabolic cages for 24 hours with free access to food and water. Blood samples of 0.3 ml were taken by puncturing a tail vein under ether anesthesia at regular intervals starting at day 0. Sera were frozen and stored at −20 C until all samples from each rat could be analyzed simultaneously for the presence of anti-glomerular basement membrane (anti-GBM) antibodies. Body weights were determined weekly.

At days 4, 8, 13, 21, and 40, four animals injected with mercury and one control animal were killed. Five control animals were killed simultaneously at day 0. The spleen, all cervical and renal lymph nodes, thymus, patches of Peyer, kidney, submandibulary salivary gland, and tongue were dissected. Samples of these organs were snapfrozen in precooled isopentane at −70 C; samples from the same organs were processed for routine histology after metacrylate embedding. Electron microscopy was performed on kidney cortex tissue, which was fixed by immersion in 1.5% glutaraldehyde and 1.0% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, as described.[15] The spleen, cervical and renal lymph nodes were initially weighed and, after samples for histology had been taken, single cell suspensions were made from the remaining tissue.

Antisera

Mouse monoclonal antibodies (MAbs) were used to phenotype leukocytes and their specificities are listed in Table 1. The MAbs MARM-4, MARD-3, MARG1-1, MARG2b-8, MARG2c-2, MARA-2, and MARE-1, directed against rat IgM, IgD, IgG1, IgG2b, IgG2c, IgA, and IgE, respectively, were purchased from Serotec (Blackthorn, UK), as well as a polyclonal sheep antiserum directed against rat IgG subclass 2a. A polyclonal rabbit antiserum directed against the glycolipid Asialo GM1 was obtained from Wako Chemicals GmbH (Düsseldorf, FRG). This antiserum recognizes natural killer (NK) cells and to a lesser extent T lymphocytes, monocytes, and polymorphonuclear leukocytes.[27,28] For each batch of antisera the optimal saturating dilution was determined in the appropriate assay.

Anti-GBM Antibody Assay

Rat GBM was isolated essentially as described by Bowman et al.[29] Briefly, glomeruli were obtained from normal BN rats by differential sieving and centrifugation of minced kidney-cortices. The glomerular suspension was sonicated, washed, and lyophilized. The GBM was collagenase-digested, using E.C. 3.4.24.3 (Sigma) at 0.7% wt/wt for 1 hour at 37 C.

Rat GBM (100 μl of 10 μg/ml in 0.1 M sodium carbonate buffer [pH 9.6] per well) was coated to Titertek PVC microtiter plates (Flow Laboratories, Herts, UK). After washing, post-coating with bovine serum albumin (Sigma), and washing, wells were incubated with 100 μl test serum, which was optimally diluted to 1:100. Wells were washed and incubated with 100 μl horseradish peroxidase (HRP; Sigma)-conjugated rabbit anti-rat Ig, diluted 1:1000. After washing, peroxidase activity was developed with H_2O_2 and orthophenylenediamine (Sigma); the reaction was stopped after 10 minutes by adding 50 μl 2 M H_2SO_4 per well. Reaction product was quantitated by measuring extinction at 492 nm using a Titertek Multiscan MC (Amstelstad, Zwanenburg, The Netherlands). Each serum was assayed in triplicate. Samples of a serum pool of untreated BN rats and of pooled sera of BN rats, which were treated with mercuric chloride and were bled at day 14, served as negative and positive controls respectively. Nonspecific binding to wells, which had not been coated with GBM, was similar for positive and negative control sera when diluted 1:100, and always less than binding of negative control sera to GBM-coated wells. Results are expressed as percentages of binding obtained with samples from the positive control serum.

Immunohistochemistry

Immunoglobulin binding to renal antigens was studied by direct immunofluorescence on acetone-fixed 2-μ cryostat sections. Total rat Ig was detected with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat Ig (Nordic, Tilburg, The Netherlands). To determine the rat Ig isotype, sections were incubated with mouse MAbs or sheep polyclonal antiserum specific for the different isotypes; after rinsing, the sections were incubated with FITC-conjugated affinity-purified goat anti-mouse IgG plus IgM heavy and light chains (Kirkegaard and Perry Laboratories, Gaithersburg, MD) or FITC-conjugated swine anti-sheep IgG (Serotec), respectively. These secondary antisera were supplemented with 1% vol/vol normal rat serum to inhibit crossreactivity of the conjugated antibodies with rat tissue. As a control for crossreactivity, the mouse monoclonal antibody RC2, directed against a human renal carcinoma antigen and not reacting with rat tissue,[30] or normal sheep serum, respectively, were used as primary antisera. The sections were rinsed and embedded in 90% vol/vol glycerol in PBS, pH 8.0, containing 25 mg/ml 1,4-diazobicyclo-(2,2,2)-octane (DABCO; Janssen Chimica, Beerse, Belgium) to inhibit fading of the fluorochrome during fluorescence microscopy.[31]

Lymphoid and nonlymphoid organs were studied for the composition of leukocyte populations and the expression of MHC antigens by a two-step immunoperoxidase technique as described.[25] Briefly, air-dried and acetone-fixed 6-μ cryostat sections were incubated with sera listed above and subsequently with HRP-conjugated second-step antisera (Dakopatts, Glostrup, Denmark). Crossreactivity of secondary antisera was inhibited and controlled as described for immunofluorescence.

Coded sections were independently examined by two observers. The number of positive cells per glomerulus in the 6-μ kidney sections was counted in 53 $\pm$ 13 glomeruli per rat. Numbers obtained by this method have been shown to correlate well with the number of positive cells counted in intact glomeruli isolated from the same kidney.[32]

Cell Suspensions

Single cell suspensions of spleen, and cervical and renal lymph nodes were prepared on ice by mincing with scissors in RPMI 1640 (Flow), supplemented with 1% wt/vol BSA and 15 mM NaN_3, and differential sieving. The suspensions were washed twice and resuspended in medium to 2.10^7 leukocytes per ml, at 4 C. Viability of the cells, as determined by trypan blue dye exclusion and phase contrast microscopy, was always higher than 90%.

Fifty microliter aliquots of each suspension were incubated with 50 μl FITC-conjugated mouse MAbs for 30 minutes at 4 C. After washing twice, the suspensions were analyzed on a FACS II (Becton Dickinson, Mountain View, CA), equipped with an argon laser (Spectraphysics, Eindhoven, The Netherlands) at 488 nm. At least 10,000 cells were analyzed. Forward light scatter windows were used to exclude erythrocytes and dead cells. Scatter and fluorescence signals were analyzed with a Nuclear Data ND-100 pulse height analyzer, interfaced with a PDP 11 computer system (Digital, Maynard, MA) for data storage and analysis.

Cells from another sample of each suspension were cytocentrifuged as described.[33] Cytocentrifuged cells were fixed in precooled 5% vol/vol glacial acetic acid in absolute ethanol for 12 minutes on ice, and washed

Table 2—Weights of Body and Secondary Lymphoid Organs During $HgCl_2$-induced Autoimmune Syndrome in the BN Rat

Day	N	Body (g)	Spleen (mg)	CLN (mg)	RLN (mg)
0	5	302 ± 37*	514 ± 37	225 ± 70	22 ± 14
4	4	248 ± 22	560 ± 10	199 ± 38	29 ± 13
8	4	260 ± 16	788 ± 129†	478 ± 71†	98 ± 27†
13	4	271 ± 27	1234 ± 87†	1222 ± 197†	140 ± 37†
21	4	249 ± 36	948 ± 98†	725 ± 75†	62 ± 29
40	4	193 ± 19†	619 ± 102	212 ± 67	22 ± 9

* Results are expressed as mean ± SD.

† $P < 0.05$ when compared with day 0 by one-way analysis of variance using Tukey's multi-comparisons procedure.

three times with ice-cold PBS. To detect both cytoplasmic and surface Ig, cytospots were incubated with tetra rhodamine isothiocyanate (TRITC)-conjugated affinity-purified goat anti-rat IgG heavy and light chains (Kirkegaard and Perry Laboratories) for 1 hour at room temperature. After rinsing three times in PBS the slides were mounted in glycerol/PBS/DABCO as described above. At least 300 leukocytes from each sample were examined by epifluorescence with an Orthoplan (Ernst Leitz, Wetzlar, FRG).

Statistical Analysis

Data obtained from flow cytometry on cell suspensions and fluorescence analysis of cytospots were statistically evaluated by one-way analysis of variance, using the multicomparisons procedure of Tukey; all possible pairs were tested. The Wilcoxon two-sample test was used to compare the numbers of MAb-positive leukocytes per glomerulus of the $HgCl_2$-injected rats with those of the control rats.

Results

Proteinuria and Anti-GBM Autoantibodies

During the course of this study one of 26 mercury-treated animals died at day 3 and, after day 20, three of eight animals at days 21, 28, and 39, respectively. From day 20, all mercury-treated rats deteriorated, as shown by weight loss (Table 2), ruffled fur, and in some cases dermatitis and conjunctivitis.

Proteinuria and serum levels of anti-GBM antibodies are depicted in Figure 1. Increased proteinuria occurred in two phases: a short transient bout of proteinuria immediately after the first injection of $HgCl_2$ followed by nephrotic range proteinuria starting at day 11 and declining after day 16, with an average maximum of 640 mg per 24 hours. Proteinuria in surviving animals eventually declined to background levels (not shown).

Increased anti-GBM antibody concentration in serum was detected from day 8 by ELISA. From day 13 on, serum levels declined, in a similar pattern as described for proteinuria.

With direct immunofluorescence, rat IgM, IgG1, and IgG2a were found to be fixed in a linear pattern along the glomerular and tubular basement membranes at day 13. In the glomeruli, this pattern appeared to be changed to mixed linear and granular at days 21 and 40 for IgG1 and IgG2a, but IgM-binding remained linear. Electron microscopy revealed subepithelial electron-dense deposits at days 21 and 40. In kidneys from control animals some rat Ig was found in the mesangium and in peritubular capillaries, but never along the basement membranes. Effacement of podocytes was assessed by electron microscopy in the mercury-treated rats from day 13 and was still present at day 40.

Changes in Lymphoid Organs

Secondary lymphoid organs transiently increased in weight and cellularity, starting between day 4 and day 8 (Table 2). Maximum levels were observed at day 13 as described for proteinuria and anti-GBM antibody serum levels. The increase in weight was more pronounced for cervical and renal lymph nodes than for the spleen and occurred earlier for renal lymph nodes than for cervical lymph nodes and spleen.

In contrast with secondary lymphoid organs, the thymic cortex became progressively depleted of lymphocytes, as assessed by histology. This depletion was first noted at day 4.

Analysis of Cell Suspensions

To characterize the quantitative changes in lymphoid organs FACS-analysis was performed on

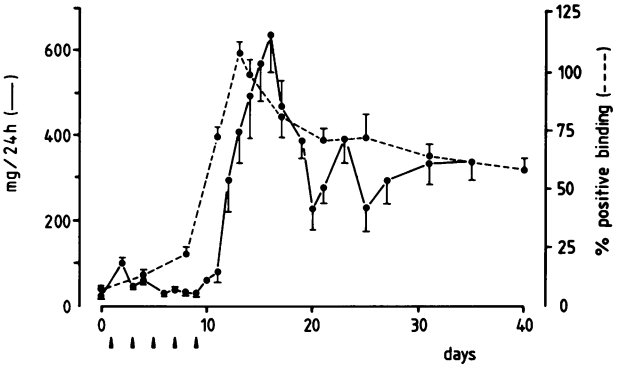


Figure 1—$HgCl_2$-induced proteinuria (——) and serum anti-GBM antibodies (- - -) in the BN rat. $HgCl_2$ (1 mg/kg body weight) was injected subcutaneously at days 1, 3, 5, 7, and 9. Each point represents values of at least four animals. Values are expressed as mean ± SEM.

spleen, and cervical and renal lymph node cell suspensions from days 0, 4, 8, 13, 21, and 40, incubated with ER13, OX19, ER2 or OX8, and compared with fluorescence microscopy on fixed cytospots stained with anti-rat Ig. The composition of each lymphoid organ was studied by analysis of both cell suspensions and immunohistochemistry on tissue sections; to obtain an indication of the absolute numbers of the diverse cell populations studied, the relative numbers were corrected for changes in organ weight. In separate experiments positive correlations were found between organ weight and cell number (for spleen $r = 0.68$; $P < 0.01$). The size of the renal lymph nodes at day 0 did not allow a full analysis. Results are summarized in Figures 2 and 3.

In all secondary lymphoid organs an increase of ER13+ MHC class II antigen-bearing cells was observed, starting between days 4 and 8, and reaching its maximum at day 13 (Figure 2). This was paralelled by an increase of blastoid cells, which were negative for OX19, OX8, and ER2 and for the B lymphocyte markers HIS22 and HIS24 (not shown). The blastoid cells could be identified as plasmablasts and plasmacells by fluorescence microscopy after staining with TRITC-conjugated goat anti-rat IgG heavy and light chains. At day 13, approximately 50% of cervical lymph node cells were plasmablasts or plasmacells, compared with 2% at day 0; in the spleen plasmacells increased from 2% at day 0 to 13% at day 13 (Figure 3).

Both OX19+ and ER2+ cells showed an early increase in the spleen, starting between days 0 and 4, and reached maximum levels at day 13. In the lymph nodes the increase of OX19+ and ER2+ cells started after day 4, as reflected by the cell numbers corrected for organ weight; again a transient pattern was found with maxima at day 13. OX8+ cells increased from day 4 in peripheral lymphoid organs, especially in the renal lymph nodes. Maximum levels were found at day 8 in the spleen and the renal lymph nodes and at day 13 in the cervical lymph nodes. At day 40, all lymphoid subsets studied had returned to baseline levels (Figure 2).

Histologic Analysis of Lymphoid Organs

The spleen showed an enlargement in white pulp after day 8 caused by the expansion of the periarteriolar lymphocyte sheath (PALS) areas and the development of HIS14+ follicles with large HIS22− centers. At day 13, these germinal centers were most pronounced, containing IgM-, IgG1- and IgE-bearing B-lymphocytes. In the red pulp an increased number of plasmacells was found containing IgM, IgG1, IgG2a, IgG2b, or IgE. Deposits of IgM, IgG1, and IgG2a were present in splenic capillaries; this also was seen along the glomerular basement membrane at day 13. A marked increase of HIS17+ and OX8+ cells was noted in the PALS at day 13, but OX19+ cells had decreased. From day 21 to day 40 the splenic architecture returned to that seen in the control animals.

After injection of mercuric chloride, a strong germinal center reaction also was observed in cervical and renal lymph nodes. The number of IgG1+, IgG2a+ and IgE+ germinal centers increased; few IgG2b or IgA containing centers had been found at day 13. Paracortical and medullary areas were markedly expanded. Hematoxylin and eosin (H & E) staining revealed many blastoid cells and mitotic figures. The paracortex contained an increased number of OX8+ lymphocytes at day 13, especially in the renal lymph nodes. The medulla mainly consisted of HIS17+ cells, which were negative for OX19, OX8, ER2, HIS14, and anti-Asialo GM1. Many IgG1- or IgE-containing plasmacells appeared to be present in the medulla (Figure 4). As in the spleen much cellular debris was present in the lymph nodes at day 21; this coincided with an increased expression of the macrophage determinants recognized by ED1 and ED2, compared with day 13.

All germinal centers in the patches of Peyer contained IgE at day 13, in contrast with day 0, when no IgE could be detected. No overt changes in the T lymphocyte population were noted by immunohistochemistry during the course of the autoimmune reaction.

In the thymic cortex fewer thymocytes were found between the MHC class II expressing epithelial cells at day 4, compared with day 0 (Figure 5). The density of cortical epithelial cells and ED2+ macrophages within a microscopic field had clearly increased. The relative volume of the cortex decreased. Depletion of the thymic cortex progressed from day 4 on; at day 40, virtually no lymphocytes were found in the epithelial matrix. The condensing cortical epithelium remained OX6+ and OX17+ throughout the whole observation period (Figure 5). During this depletion, HIS14+ follicles with germinal centers developed in the thymic medulla.

Changes in Nonlymphoid Organs

Kidney

In the normal kidney, MHC class II antigens were expressed by epithelial cells of proximal tubules, by scattered interstitial cells often with slender cell processes, and by some mesangial cells and passenger leu-

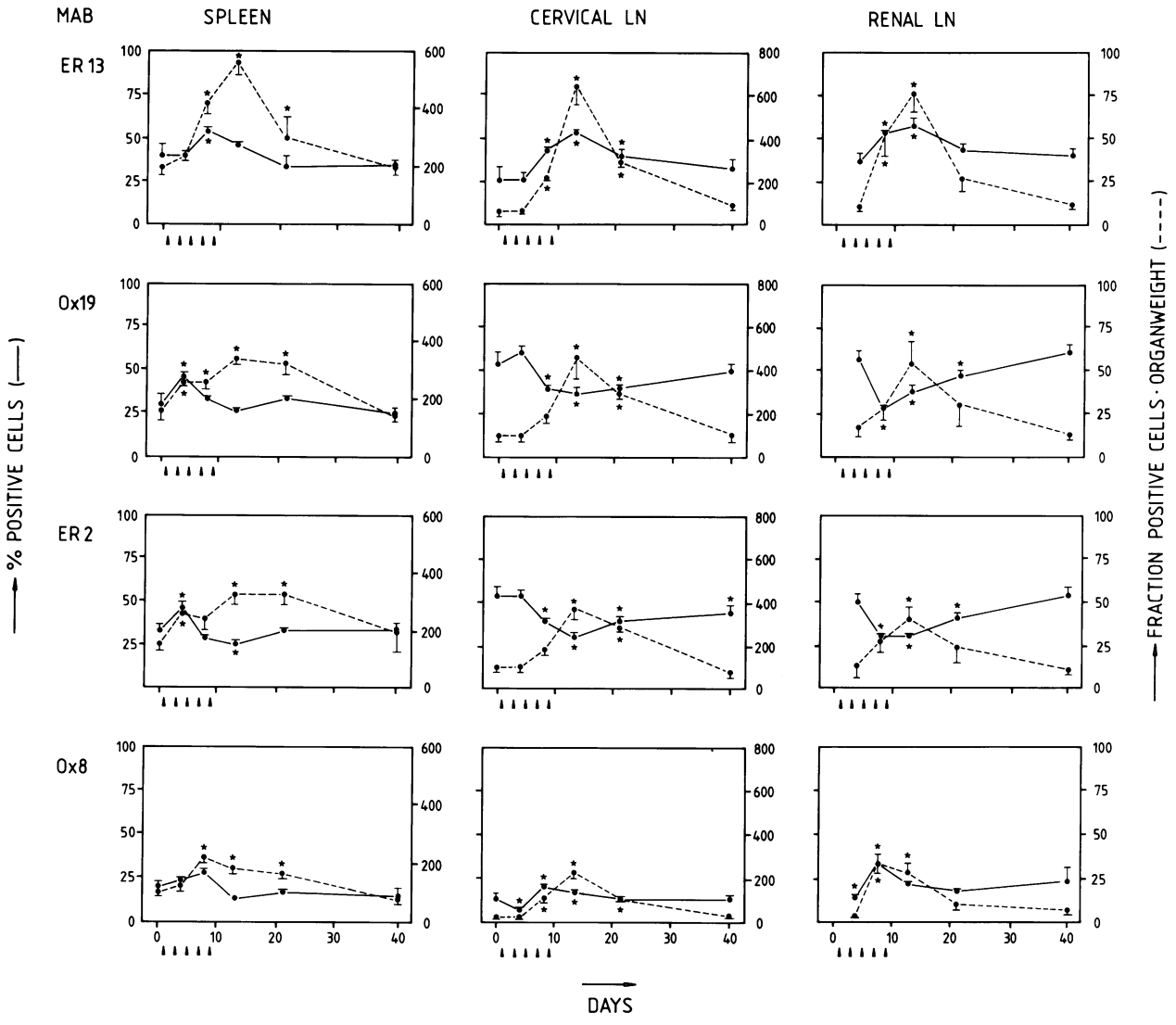


Figure 2—Frequency of ER13, Ox19, ER2, and Ox8 binding cells in the spleen, and cervical and renal lymph nodes during $HgCl_2$-induced autoimmune syndrome in the BN rat. $HgCl_2$ (1 mg/kg body weight) was injected subcutaneously at days 1, 3, 5, 7, and 9. Flow cytometry was performed on single cell suspensions obtained at days 0 (N = 5), 4, 8, 13, 21, and 40 (all N = 4). On the left y-axis the percentage positive cells after correction for binding with MAb, not directed against rat tissue, is indicated; on the right y-axis the product of the fraction positive cells and the organ weight is expressed. Values are given as mean ± SD. Asterisks indicate significant differences with day 0 for spleen and cervical lymph node cells and with day 40 for renal lymph node cells; $P < 0.05$ by one-way analysis of variance with Tukey's multicomparisons procedure.

kocytes (Figure 6). No difference was found between OX6 and OX17 positivity.

At day 4, the proximal tubule epithelial cells of the pars recta appeared to be damaged by the direct toxic effect of mercuric chloride and had lost MHC class II antigens. At day 8 these tubules showed many mitotic figures, indicative for regeneration. No clear differences in MHC class II expression by proximal tubules were detected from day 8 compared with day 0. After day 4 more dendritic interstitial cells positive for OX6, OX17, 1F119, HIS17, and ER2 and negative for all other antibodies used were present. At day 8, perivascular infiltrates developed, which were more pronounced at day 13. Many interstitial infiltrates between tubules and around Bowman's capsule were seen (Figure 6). At day 13, these infiltrates mainly consisted of MHC class II antigen-bearing T lymphocytes and monocytes; because of overlapping numbers, the presence of CD4+CD8+ T lymphocytes can be deduced (Table 3). The epithelium lining calyx and pelvis was also heavily infiltrated with MHC class II positive leukocytes. Interstitial infiltrates diminished from day 21, but were still present at day 40, as were perivascular infiltrates. Throughout the observation period no clear changes were noted in the expression of MHC class I antigens, except for the presence of OX18+ infiltrating leukocytes.

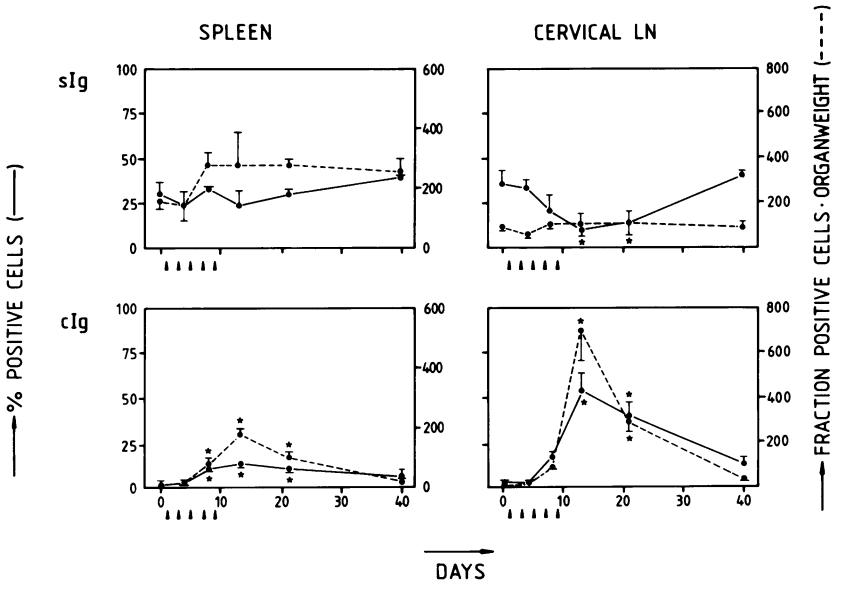


Figure 3—Frequency of surface Ig-bearing cells (sIg) and cytoplasmic Ig-containing cells (cIg) in spleen and cervical lymph nodes during $HgCl_2$-induced autoimmunity. Ig was detected by epifluorescence of fixed cytospots, and incubated with TRITC-conjugated goat anti-rat IgG, heavy and light chains. Analysis was performed on the same cell suspensions as used for flow cytometry (Figure 2). Values are expressed as in Figure 2.

Glomeruli were investigated for the presence of cells positive for OX6, OX17, OX19, ER2, OX8, ED1, or Asialo GM1 by counting the number of positive cells per glomerulus in 6-μ sections for approximately 50 glomeruli per rat. Results are given in Table 4. Significant differences compared with day 0 were found at day 4, when the number of ED1+ monocytes had increased from 0.8 to 2.7 per glomerulus per section, and at day 13, when 0.8 OX8+ cell per glomerulus per section were present *vs.* 0.3 at day 0. ED1+ and Asialo GM1+ cell numbers did not differ from day 0; ER2+ cells decreased.

Salivary Glands and Tongue

Both the salivary glands and tongue contained few MHC class II antigen-expressing cells at day 0 (Figures 7a and 8a). After administration of $HgCl_2$, a gradually increased expression of MHC class I and II antigens by mainly ductal epithelium was observed in the salivary glands, becoming maximal at day 13. Acinar epithelium showed increased class I, but not class II expression (Figures 7b and 7d). In the tongue, an increased expression of MHC class I antigens by epidermal cells was found at day 13, but had not yet occurred at day 8. Epithelial cells also showed increased class II expression focally (Figure 8b).

Concomitant with increased expression of MHC antigens, infiltrates of leukocytes appeared perivascularly and predominantly adjacent to the MHC class II positive epithelium. At day 13, leukocytes could also be found between epithelial cells (Figure 8). The infiltrates consisted of MHC class II positive, HIS17+, OX19+, ER2+, OX8+, ED1+, and, in the tongue, but less in salivary glands, anti-Asialo GM1+ leukocytes. After day 13, MHC antigen expression by non-lymphoid tissue decreased and the infiltrates gradually disappeared.

Discussion

The determinants recognized by ER2 and OX8 will be designated as CD4 and CD8, respectively, when appropriate.

A marked proliferation of both B and T lymphocytes was observed in peripheral lymphoid organs by flow cytometry on single cell suspensions and by immunohistochemistry.

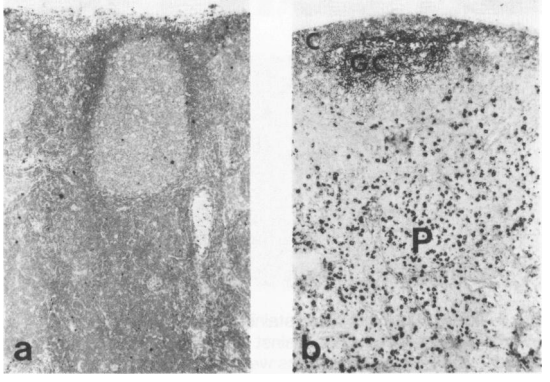


Figure 4—Immunoperoxidase staining of cryostat sections of cervical lymph nodes from a normal rat (**a**) and from an $HgCl_2$-treated rat at day 13 (**b**), using the MAb MARE-1 against rat IgE. $HgCl_2$ induced the development of germinal center (GC) and plasmacellular (P) reactions involving the IgE isotype; IgE is also present on corona B lymphocytes (C). ×34

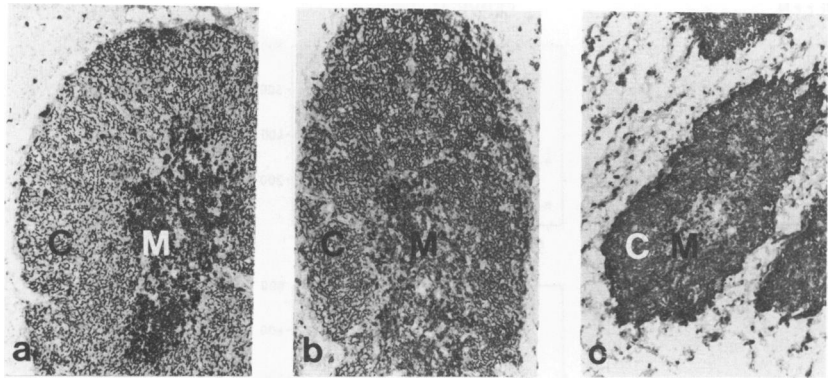
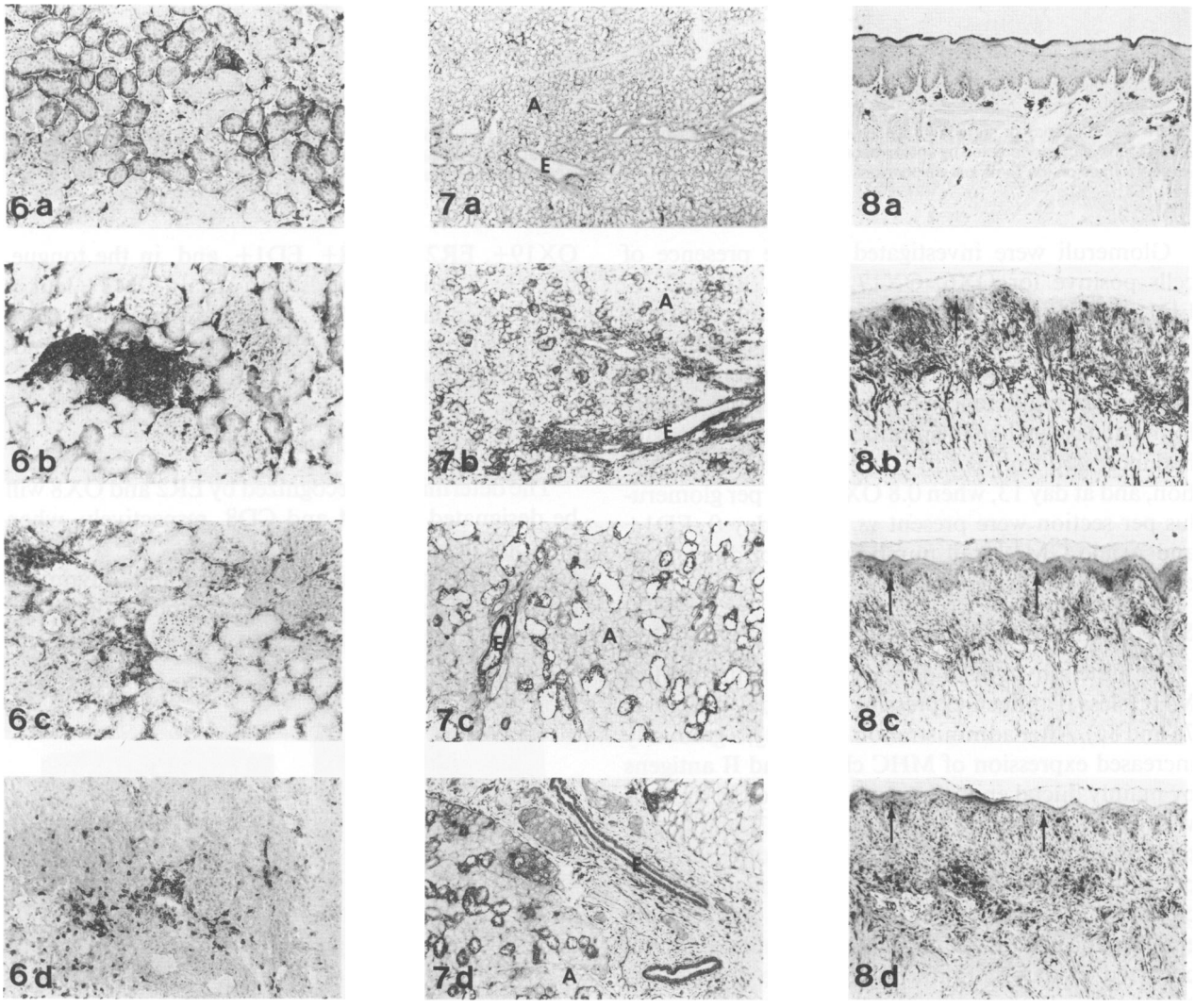


Figure 5—Immunoperoxidase staining of cryostat sections of thymi from a normal rat (**a**) and from $HgCl_2$-treated rats at day 4 (**b**) and day 40 (**c**), using MAb OX6 against MHC class II antigen. After $HgCl_2$ administration, the thymic cortex (C) is progressively depleted of lymphocytes, resulting in condensation of the cortical epithelial matrix; M, thymic medulla. ×69



Figures 6–8—Immunoperoxidase staining of cryostat sections of the kidney (6), salivary gland (7), and tongue (8) of normal or mercury-treated rats. The following MAbs were used: OX6 against MHC class II antigen, OX18 against MHC class I antigen, ER2 against CD4, and OX8 against CD8. **Figure 6**—In normal kidneys MHC class II antigens were expressed by scattered interstitial cells, proximal tubular epithelium, and some passenger leukocytes and mesangial cells (**a**). At day 13 MHC class II positive perivascular and interstitial infiltrates developed (**b**), consisting of CD4+ (**c**) and CD8+ leukocytes (**d**). Many MHC class II and CD4 positive interstitial dendritic cells were present at day 13 (6b, 6c). ×55 **Figure 7**—Mercury administration induced increased expression of both MHC class I (**d**) and class II (**b**) antigens in salivary glands by mainly epithelial cells at day 13, compared with MHC class I (**c**) and MHC class II (**a**) antigen expression in controls. Large infiltrates of MHC class I and class II antigen positive leukocytes were present at day 13 (7b, 7d). A, acini; E, excretory duct. ×34 **Figure 8**—In the tongue large infiltrates of MHC class II positive leukocytes were present at day 13 (**b**) compared with controls (**a**). MHC class II positive (b), CD4+ (**c**), and CD8+ (**d**) leukocytes could be found in the stratum basale of the epithelium (arrows). ×43

Table 3—Composition of Renal Perivascular and Interstitial Infiltrates in $HgCl_2$-treated BN Rats at Day 13

Cells	Percentage positive cells at day 13
OX6+	90–100
OX17+	90–100
ER2+	70–80
OX8+	30–50
ED1+	40–60
HIS14+	10–20
Asialo GM1+	5–10

The proportions of the various lymphocyte subpopulations in spleen and lymph nodes at day 0 (Figures 2 and 3) agree in general with those reported for normal PVG/c, AO or DA rat strains.[20,25] It should be noted, however, that in this study the sum of ER2+ and OX8+ cells always exceeded the observed number of OX19+ cells. This may be due to the reactivity of ER2 and OX8 with non-T leukocytes.[21–23] The appearance of CD4+CD8+ lymphocytes, as hypothesized below, may also contribute to this phenomenon. Nevertheless, changes in the sum of ER2+ and OX8+ cells are in general reflected well in changes in the number of OX19+ cells (Figure 2), indicating that these changes do concern T lymphocytes.

The first changes in the periphery were found in the spleen. At day 4, an increase of CD4+ lymphocytes was seen by flow cytometry.

At days 8 and 13 strong germinal center and plasmacellular reactions became apparent, not only in the spleen and lymph nodes but also in the patches of Peyer and in the thymic medulla. The IgG1 and IgE isotypes were predominantly involved in these reactions; with direct immunofluorescence of the kidney, IgE was not observed among glomerular-bound antibodies, confirming previous findings.[3,4]

Concomitant with the generation of plasmablasts, an increase of MHC class II antigen-bearing blastoid cells was observed in the spleen and lymph nodes (Figure 3). The B lymphoproliferation probably accounts for this increase of MHC class II positive cells. Flow cytometry did not indicate the presence of MHC class II antigen-bearing T lymphocytes in peripheral lymphoid organs; however, double staining was not performed and their presence cannot be excluded.

In addition to B lymphoproliferation, the total T lymphocyte population of both CD4 and CD8 phenotypes showed an absolute increase in peripheral lymphoid organs. Autoreactive T cell clones directed against MHC class II antigens have been shown to induce polyclonal B cell responses of the IgM isotype in an MHC-unrestricted way and to help antigen-specific IgG responses, which were strongly MHC restricted, both *in vitro*[34,35] and *in vivo*.[35] Furthermore, T cell clones that can induce both IgG and IgE synthesis in normal B lymphocytes were suggested to be directed against self MHC class II antigens.[36] *In vivo* helper activity of anti-MHC class II autoreactive T clones to Tc lymphocytes also has been demonstrated.[37] In view of the MHC class II dependent T–T cell interaction in the $HgCl_2$ model,[8] a role for anti-MHC class II autoreactive T cells in mercury-induced autoimmunity is suggested.

It is tempting to speculate that the increasing CD4+ T lymphocytes in the spleen at day 4 comprise such cells, which may in turn activate both B and T lymphocytes in a more or less polyclonal way, accounting for the observed peripheral lymphoproliferation at days 8 and 13 and leading to the emergence of autoreactive effector lymphocytes. Taking this view, the CD8+ leukocytes at day 8, increasing simultaneously with the plasmablasts, might be effector Tc lymphocytes; interestingly, after the proliferation of CD8+ cells in the lymphoid organs, CD8+ cells were found in the infiltrates in target organs.

Peripheral down-regulation of autoreactive T lymphocytes, which have escaped elimination in the thymus, has been shown to be mediated by Ts lymphocytes.[38–41] Mercury might interfere with Ts lymphocyte function in an early stage of the syndrome, allowing proliferation of autoreactive T lymphocytes, as suggested by earlier studies in the PVG/c rat[42] and the BN rat.[43] Clearly, functional assays have to be performed to determine the nature of the increasing CD8+ cells at day 8.

The role of the thymus in this model is intriguing. The cortical depletion does not occur in $HgCl_2$-treated Lewis rats (unpublished results). These rats do not develop the autoimmune syndrome after $HgCl_2$ administration,[1] although their proximal tubules are directly damaged to an extent similar to damage in BN rats. Therefore the depletion of the thymic cortex is probably not caused by stress. Cortical depletion in the BN rat had already started before day 4. It is possible that autoreactive T lymphocytes or their precursors are able to leave the thymus on $HgCl_2$ administration, because the normal intrathymic elimination of autoreactive T clones is hampered.[44] This might be due to interference with Ts lymphocyte function by $HgCl_2$ similar to that discussed above for peripheral Ts lymphocytes.

The nature of the relation between cortical depletion and the development of $HgCl_2$-induced autoimmunity remains to be established. It is clear, however, that an intact thymus is not a prerequisite for this syndrome, because adult thymectomy in the PVG/c rat does not prevent the development of autoimmune glomerulopathy after $HgCl_2$ administration.[42]

Table 4—Glomerular Leukocytes During $HgCl_2$-induced Nephritis

Cells	No. of cells per glomerular section (day)*					
	0 (N = 8)	4 (N = 3)	8 (N = 3)	13 (N = 3)	21 (N = 3)	40 (N = 3)
OX6+	1.4 ± 0.2†	2.0 ± 0.8	1.3 ± 0.2	1.2 ± 0.3	1.3 ± 0.2	1.2 ± 0.2
OX17+	1.5 ± 0.4	1.8 ± 0.0	1.2 ± 0.1	1.1 ± 0.2	1.5 ± 0.2	1.1 ± 0.1
OX19+	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.1 ± 0.0
ER2+	1.2 ± 0.2	1.1 ± 0.2	0.7 ± 0.2	0.5 ± 0.2‡	1.0 ± 0.1	0.7 ± 0.1‡
OX8+	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.8 ± 0.3‡	0.4 ± 0.1	0.4 ± 0.1
ED1+	0.8 ± 0.2	2.7 ± 0.0‡	1.1 ± 0.8	1.2 ± 0.4	1.5 ± 1.0	1.1 ± 0.5
Asialo GM1+	0.6 ± 0.3	1.9 ± 1.2	0.9 ± 0.4	1.1 ± 0.2	1.0 ± 0.1	0.8 ± 0.1

* From each rat the number of MAb-positive cells per glomerulus was counted in 53 ± 13 glomeruli in 6-μ kidney sections, stained by immunoperoxidase.
† Results are expressed as mean ± SD.
‡ $P \leq 0.01$, Wilcoxon two-sample test when compared with day 0.

The transient character of almost all $HgCl_2$-induced autoimmune phenomena is also seen when mercury administration is prolonged.[45] Both suppressor T lymphocytes and auto-anti-idiotypic antibodies have been shown to be operative in rats in remission phase, ie, when anti-GBM antibody serum levels are decreasing, and are likely to play a role in the self-limiting character of this syndrome.[45,46] The present data do not show an increase of CD8+ cells from day 13, but the levels at day 21 are significantly higher in the spleen and cervical lymph nodes compared with day 0; at this stage, the CD8+ population will at least partly consist of Ts lymphocytes. This again stresses the need of functional assays at days 8 and 13.

This study clearly shows that in mercury-induced autoimmunity the kidney is not the only target organ. In most nonlymphoid organs studied, infiltration of MHC class II antigen-bearing leukocytes was seen concomitant with or followed by an increased expression of MHC antigens by epithelium. Epithelial cells were not observed to express more MHC antigens before infiltrating leukocytes were noted. Increased expression of MHC antigens by nonlymphoid tissue in the kidney was not detected but it cannot be excluded; the immunohistochemical technique is not sensitive enough to detect an increased expression by tissue, which is already strongly positive in control animals.

Increased MHC antigen expression by nonlymphoid tissue has been described extensively and is most often associated with immunologic stimuli, for instance in graft-*vs.*-host disease.[47,48] It has been shown clearly that MHC antigen expressing nonlymphoid cells can present antigen to T lymphocytes effectively.[49,50] Although the increased expression of MHC antigens by nonlymphoid tissue is not a primary event in mercury-induced autoimmunity, it may play an important secondary role in the pathogenesis of this syndrome. Autoreactive Tc cells might recognize autoantigen in the context of MHC antigen and damage the autoantigen-presenting cells, as has been demonstrated for experimental autoimmune encephalomyelitis.[51] It is also possible that the MHC antigens are target structures for autoreactive Tc lymphocytes.[52]

The majority of the infiltrating cells in the kidney at day 13 consisted of MHC class II antigen-bearing activated T lymphocytes and monocytes (Table 3). Because the T lymphocytes observed in the peripheral lymphoid organs seemed to be MHC class II negative, it is hypothesized that these cells became positive at the site of infiltration in the target tissues.

The leukocytes, which were present between epithelial cells in the salivary gland and the tongue, were CD8+, HIS17+, MHC class II positive, and variable CD4+ and Asialo GM1+; this phenotype points to activated Tc lymphocytes.[53] Cells with a similar phenotype also might function as antibody-dependent cytotoxic cells.[28] At day 13, CD8+ cells also had increased within the glomeruli, whereas no significant increase of ED1+ monocytes or Asialo GM1+ NK cells was found (Table 4). Because the fraction of circulating CD8+ lymphocytes was reported to be decreased before anti-GBM antibody serumlevels were falling,[54] the glomerular increase of CD8+ cells is not a nonspecific reflection of the number of circulating lymphocytes. The present findings do not agree with results reported by Hinglais et al,[11] who performed an ultrastructural study of mercury-induced nephritis in the BN rat and showed an increase of monocytes around day 14 based on morphologic criteria. Using immuno-electron microscopy, CD8+ cells that resembled lymphocytes were recently detected in the glomerular capillaries. CD8+ cells also were found interstitially with extending foot processes in close contact with the tubular basement membrane (manuscript in preparation).

In addition to autoantibodies directed against basement membrane components, a pathogenetic role for CD8+ lymphocytes in mercury-induced autoimmunity must be considered and is under investigation.

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