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Neonatal induction of tolerance to T_h2-mediated autoimmunity in rats

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Abstract

Brown-Norway (BN) rats are highly susceptible to drug-induced immune dysregulations and when injected with mercuric chloride ($HgCl_2$) or sodium aurothiopropanolsulfonate (ATPS), they develop a syndrome characterized by a polyclonal B cell activation depending upon $CD4^+$ T_h2 cells that recognize self-MHC class II molecules. Since peripheral tolerance of T_h2 cells might be crucial in the prevention of immunological manifestations such as allergy, establishing conditions for inducing tolerance to $HgCl_2$- or ATPS-mediated immune manifestations appeared to be of large interest. We report here that BN rats neonatally injected with $HgCl_2$: (i) do not develop the mercury disease, (ii) remain resistant to $HgCl_2$-induced autoimmunity at 8 weeks of age and later, provided they are regularly exposed to $HgCl_2$, (iii) are still susceptible to ATPS-induced immune manifestations, and (iv) exhibit spleen cells that adoptively transfer tolerance to $HgCl_2$-induced autoimmunity in naive, slightly irradiated, syngeneic recipients. These findings demonstrate that dominant specific tolerance can be neonatally induced using a chemical otherwise responsible for T_h2-mediated autoimmunity.

Introduction

For a long time the neonatal period has been thought to be a privileged period to manipulate the immune system, and particularly to tolerize against self and non-self antigens (1). Revisiting neonatal tolerance, recent papers have demonstrated that regarding the tolerance induction, the neonatal period is rather quantitatively different from the later stages of development (2–4).

Three non-mutually exclusive mechanisms have been posited to account for acquired unresponsiveness of T cells: clonal deletion, clonal anergy and active suppression (5). In this latter case, tolerance was shown to be restored or broken by passive transfer or depletion of regulatory T cells respectively (6).

Most autoimmune disorders, either organ-specific, such as experimental allergic encephalomyelitis (EAE), or systemic, such as the MRL/*lpr* lupus model, depend upon T_h1 cells (7–9). Induction of tolerance has been well established in these autoimmune conditions by either intra-thymic injection (10–12) or by oral administration of the corresponding autoantigen with regulatory T cells being generated (13) or by passive transfer of regulatory T cells (14). By contrast, T_h2 cells appear to be much less frequently involved in autoimmune diseases. They play a role in EAE in immunocompromised mice (15), in lupus syndrome developed in B/W mice (16,17) and in allogeneic reactions (18). Moreover, T_h2 cells were first accepted as not susceptible to tolerization (19,20) and even though induction of peripheral tolerance of T_h2 cells is no longer controversial (21), a tolerant state appears more difficult to achieve in T_h2 than in T_h1 cells (22). For example, injections of parental spleen cells in F_1 neonates lead to tolerance of T_h1 cells, whereas T_h2 cells escape this phenomenon and induce autoimmunity (23,24).

Mercury- or gold-induced autoimmune disorders in the Brown-Norway (BN) strain of rats represent another example of T_h2-mediated autoimmunity. Indeed BN rats injected with mercuric chloride ($HgCl_2$) or sodium aurothiopropanolsulfonate (ATPS) develop a similar lupus-like syndrome with lymphoproliferation (25–27), hypergammaglobulinemia affecting mainly IgE and IgGl (28), production of numerous autoantibodies (against laminin, DNA, type II and IV collagen, and thyroglobulin) (26,29–31), and an autoimmune glomerulonephritis due to the deposition of anti-laminin antibodies (32–34). All the immune disorders autoregulate and thereafter animals are relatively resistant to rechallenge with $HgCl_2$

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(35,36). In humans, mercury and gold salts are also associated with the occurrence of various immune-mediated manifestations (37) and thus make the animal models valuable to study the role of environmental factors in the development of systemic autoimmunity.

For both mercury- and gold-induced immune disorders, previous studies pointed to the pivotal role of autoreactive $CD4^+$ T cells that proliferate in the presence of naive syngeneic MHC class II^+ cells (34,38,39) and induce a polyclonal B cell activation (26,34). These $CD4^+$ T cells belong to the T_h2 subset (18) and T_h2 cell lines derived from gold salt-injected rats were demonstrated to transfer the whole autoimmune syndrome in naive, CD8-depleted recipients (40). Taking advantage of this strong mediation by the T_h2 subset (34,40–43), we investigated, in this study, the conditions to induce a solid tolerance to T_h2-mediated autoimmunity. We show that neonatal injections of $HgCl_2$ in BN rats establish a solid metal-specific tolerance to $HgCl_2$-induced immune manifestations. Moreover, transfer of spleen cells from animals neonatally exposed to $HgCl_2$ is shown to protect syngeneic naive rats against mercury disease, therefore suggesting a role for an active suppressive mechanism.

Methods

Animals

BN rats, originating from the CSEAL (Orléans, La Source, France), were bred in our own animal facilities. Animals were weaned at 3 weeks of age, and were cared for and handled according to the principles expressed in the Declaration of Helsinki on the use of animals in research. Neonates and 2- to 9-month-old female and male rats were used in the following experiments.

Experimental procedure

BN rats were s.c. injected with $HgCl_2$ 3 times a week for 2 weeks at a dose of 100 μg/100 g body wt (33) starting within 24 h after birth. Control rats received the same volume of distilled water adjusted to the same pH (3.8) as the $HgCl_2$ solution, following the same schedule as for $HgCl_2$ injections. At 8–12 weeks of age, several BN rats received a second set of injections of either $HgCl_2$ or H_2O as above, or of ATPS at a dose of 2 mg/100 g body wt 3 times a week for 8 weeks, as already described (34).

Experimental groups

BN rats were neonatally injected with $HgCl_2$ (Hg rats) or H_2O rats) (first set of injections). Rats from each of these two groups were either sacrificed at 2 weeks of age or were injected, at 8–12 weeks of age, with $HgCl_2$ (Hg-Hg or H_2O-Hg rats) or H_2O (Hg-H_2O or H_2O-H_2O rats) or ATPS (Hg-ATPS or H_2O-ATPS rats) (second set of injections). In another set of experiments, BN rats neonatally injected with $HgCl_2$ were re-exposed to $HgCl_2$ either at 2, 4, 6 or 9 months of age.

Transfer experiments

Spleen cells obtained from 2- to 4-month-old BN rats neonatally injected with $HgCl_2$ or H_2O were i.v. transferred into ^{137}Cs γ-irradiated (200 rad) BN rats of 8–12 weeks of age.

Table 1. Effect of $HgCl_2$ administration in BN neonates

Neonatal injections[a]	*n*	IgE concentration (μg/ml)	Anti-laminin antibody titer (AU)[b]	Glomerular Ig deposits
H_2O	9	1.4 ± 0.6	1.3 ± 0.1	–
$HgCl_2$	9	2.3 ± 0.5	1.6 ± 0.1[c]	–

[a]Neonates received six injections of H_2O or $HgCl_2$ starting within 24 h after birth and were sacrificed at 2 weeks of age.
[b]Expressed as percent of maximum binding activity of a serum reference.
[c]Not significant using the Student's *t*-test when compared to H_2O-injected neonates.

Twenty-four hours after adoptive transfer, irradiated BN rats were exposed to 50 μg/100 g body wt of $HgCl_2$ 3 times a week as described (33).

Proteinuria and renal immunofluorescence studies

Proteinuria was assessed once a week using the biuret method and was considered as abnormal when exceeding 20 mg/24 h (33). Open wedge kidney biopsy was performed in 8- to 12-week-old rats on day 15 of the second set of injections. Kidneys were obtained after killing of 2-week-old rats on day 15 of the first set of injections or of 8- to 12-week-old rats on day 30 of the second set of injections. Kidney cryostat sections were stained with a fluoresceinated sheep antibody to rat Ig as previously described (33).

Detection of anti-laminin and anti-DNA antibodies in serum

Individual serum titers of antibodies to laminin and DNA were measured by ELISA as already described (44,45). Results were expressed as percent of maximum binding activity of a standard pool of sera originated from BN rats injected with $HgCl_2$.

Quantification of serum IgE concentration

Individual serum IgE concentrations were determined by a sandwich ELISA as follows. Microtiter plates (Maxi-Sorp; Nunc, Rocksilde, Denmark) were coated with 100 μl of the mouse monoclonal MARE antibody to the rat ε chain (Immex, Brussels, Belgium), diluted to 5 μg/ml in PBS containing 0.01% NaN_3 for 90 min at 37°C and overnight at 4°C. Rat serum samples were diluted in PBS buffer containing 0.1% gelatine and 0.01% Tween 20 (PBS gel Tw) and incubated for 2 h at 37°C. Mouse monoclonal MARK-1 antibody to the rat κ chain labeled with horseradish peroxidase (HRP) (a gift from H. Bazin, Brussels, Belgium) was used as a second antibody, diluted 1:6 000 in PBS gel Tw and incubated for 1 h at 37°C; bound HRP activity was revealed as described (44) and absorbance at 490 nm was determined with a microplate ELISA reader (MR610; Dynatech, Alexandria, VA). Results were expressed by comparison to a standard pool of BN rat sera containing known amounts of rat IgE.

Statistical analysis

Comparisons between the different groups of rats were performed using unpaired Student's *t*-test or Fisher's test as *post-hoc* procedure after ANOVA.

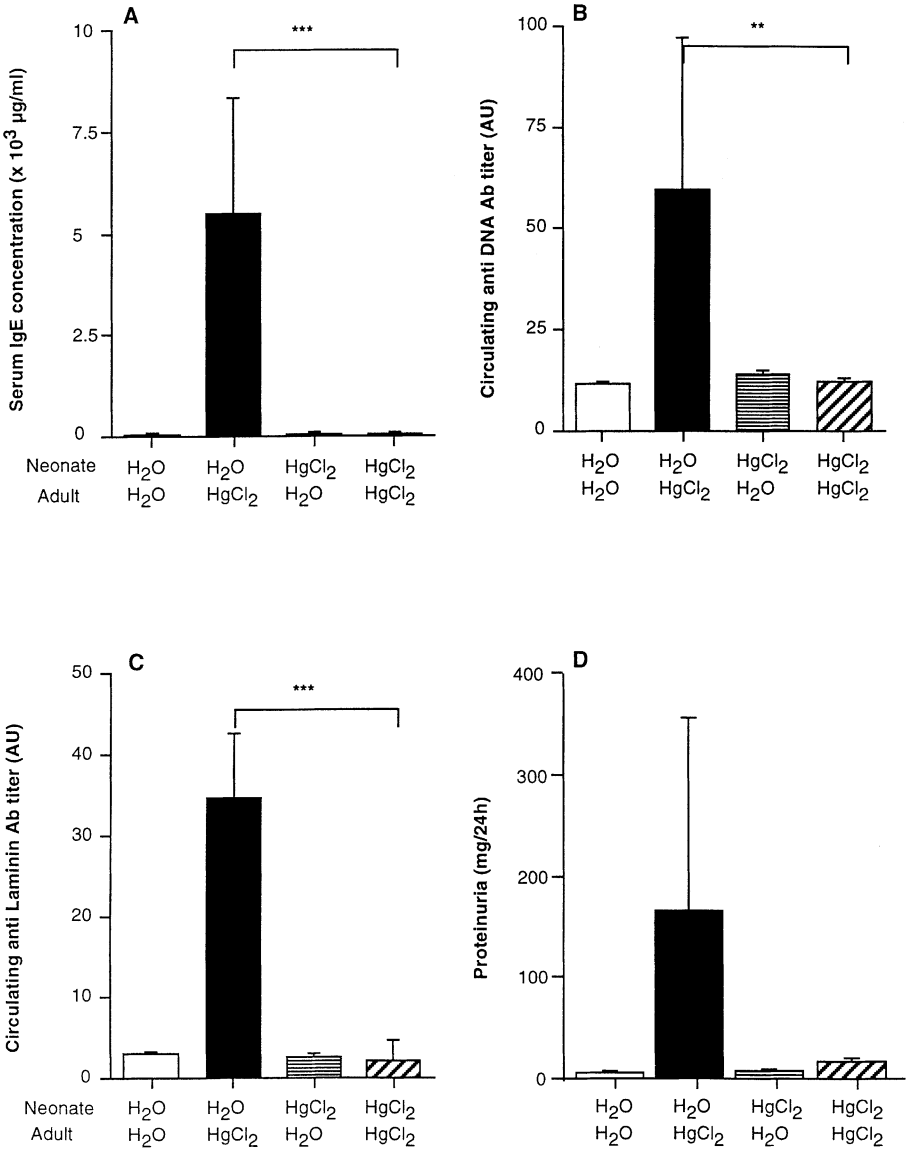

Fig. 1. BN rats neonatally injected with $HgCl_2$ are resistant to mercury disease under $HgCl_2$ exposure at 8–12 weeks of age. BN rats, when neonates, were injected with H_2O or $HgCl_2$ and then, when 8–12 weeks old (adults), were exposed to H_2O or $HgCl_2$ (see Methods). Serum IgE concentration (A), circulating anti-DNA (B) and anti-laminin (C) antibody titers were measured using specific ELISA, and proteinuria (D) was measured using the biuret method. Data represent peak values obtained during the second set of injections, i.e. in adult rats, and are expressed as the mean ± SD from 11–16 rats. Statistical analysis for Hg-Hg rats versus H_2O-Hg rats: $^{**}P<0.01$ and $^{***}P<0.001$.

Results

Neonatal injections of $HgCl_2$ make BN rats tolerant to $HgCl_2$-induced autoimmunity

Neonatally $HgCl_2$-injected BN rats sacrificed at 2 weeks of age, i.e. after six injections of $HgCl_2$, exhibited similar very low levels of circulating IgE and antibodies to laminin as neonatally H_2O-injected BN rats sacrificed at 2 weeks of age. Moreover, in both groups renal glomeruli were free of IgG deposits (Table I).

Neonatally H_2O-injected BN rats exposed to $HgCl_2$ at 8–12 weeks of age (H_2O-Hg rats) exhibited the typical $HgCl_2$-induced manifestations including a dramatic increase in serum IgE concentration (Fig. 1A) associated with the production of antibodies to DNA (Fig. 1B) and to laminin (Fig. 1C). As previously described, these manifestations peaked on day 15, then declined and were no longer observed in the third month of $HgCl_2$ administration (not shown). Moreover, on day 15 all these H_2O-Hg rats displayed typical linear IgG deposits along the glomerular capillary wall (Fig. 2a), whereas at the time of sacrifice, by the end of the second month of $HgCl_2$ administration, glomerular IgG deposits were distributed in a granular pattern along the capillary walls (Fig. 2b) and in the arteriolar walls. Finally, all of the H_2O-Hg rats developed proteinuria (Fig. 1D).

In sharp contrast, neonatally $HgGl_2$-injected BN rats exposed to $HgCl_2$ at 8–12 weeks of age (Hg-Hg rats) had similar circulating anti-DNA (Fig. 1B) and anti-laminin (Fig. 1C)

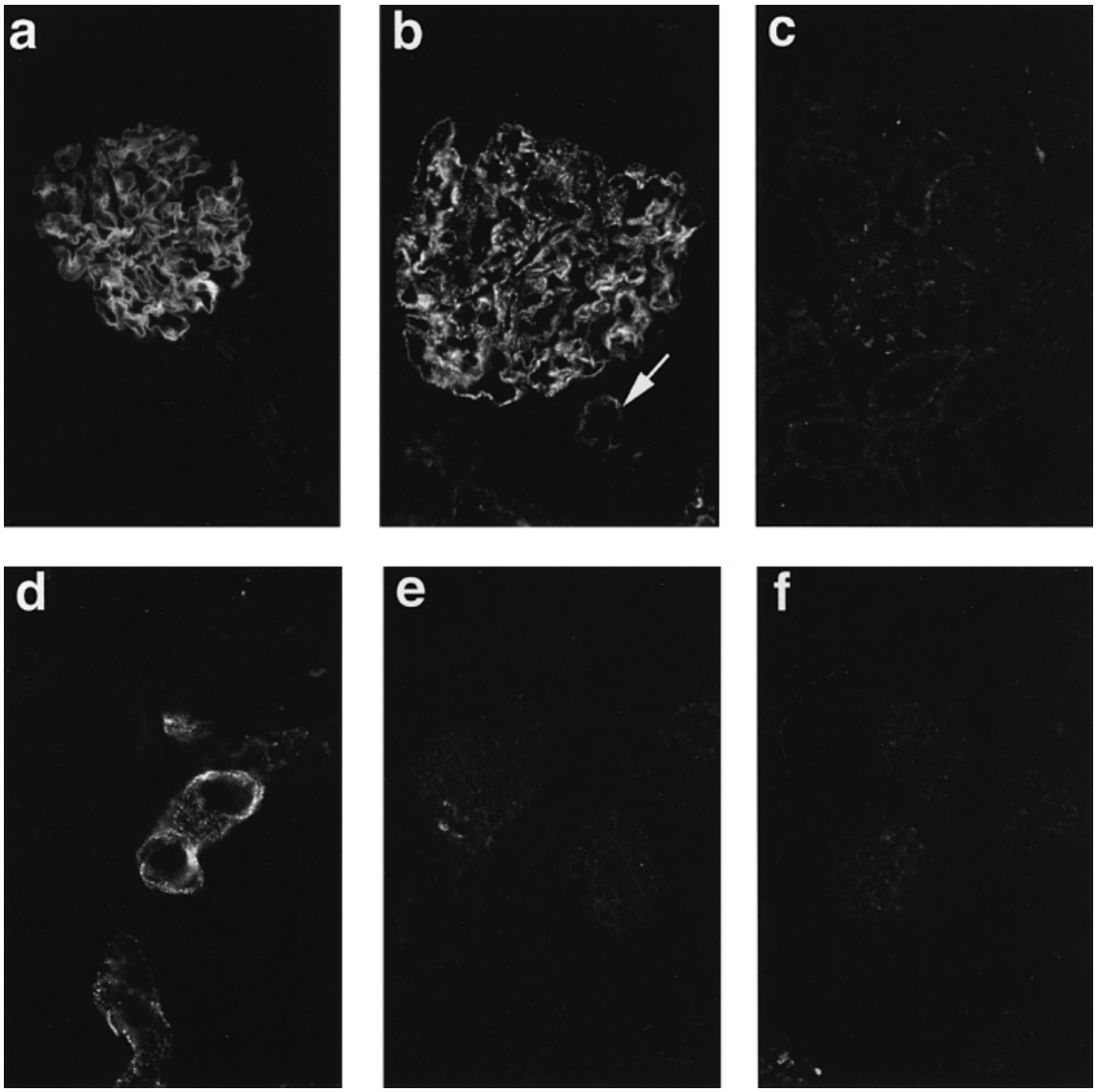

Fig. 2. Immunofluorescence studies. BN rats, when neonates, were injected with H_2O or $HgCl_2$ and then, when 8–12 weeks old (adults), were exposed to H_2O or $HgCl_2$ (see Methods). Kidney cryostat sections were stained with FITC-labeled sheep anti-rat IgG antibodies. In H_2O-Hg rats (n = 16), on day 15 of $HgCl_2$ exposure (a; original magnification ×250), IgG deposits are observed along the glomerular capillary walls in a linear pattern, and on day 60 of $HgCl_2$ exposure (b; original magnification ×250), in a granular pattern along the glomerular capillary walls and in the arteriolar walls (arrow). In contrast, in Hg-Hg rats (n = 11), only on day 60 of $HgCl_2$ exposure (c; original magnification ×160) very light granular IgG deposits are disseminated within the mesangium and (d; original magnification ×250) granular IgG deposits are seen mainly in arteriolar walls. No staining is ever seen either in H_2O–H_2O rats (n = 14) (2; original magnification ×160) or in Hg–H_2O rats (n = 12) (f; original magnification ×160).

antibody titers as Hg-H_2O or H_2O-H_2O control rats on day 15 (Fig. 1B and C) or at any time thereafter (not shown). In Hg-Hg rats serum IgE concentration significantly increased ($P<0.05$) during the second week of $HgCl_2$ exposure as compared to H_2O-H_2O rats and then plateaued (48 ± 24 versus 11 ± 4 μg/ml), but this increase was much lower ($P<0.001$) as compared to H_2O-Hg rats (48 ± 24 versus 5420 ± 3050 μg/ml). Moreover, none of the Hg-Hg rats developed proteinuria (Fig. 1D); they only displayed scarce IgG deposits in the mesangial areas (Fig. 2c) and granular IgG deposits in the arteriolar walls (Fig. 2d) at the time of sacrifice (8 weeks after starting the second set of $HgCl_2$ injections). No renal staining was ever observed either in H_2O-H_2O or Hg-H_2O rats (Fig. 2e and f).

Neonatally $HgCl_2$-induced tolerance is transient and dependent upon the presence of $HgCl_2$

BN rats neonatally injected with $HgCl_2$ received a second set of $HgCl_2$ injections at 2, 4, 6 or 9 months of age (Hg-Hg rats). As shown in Fig. 3, as compared to control BN rats that received only one set of $HgCl_2$ injections at 2 months of age, in Hg-Hg rats, serum IgE concentration (Fig. 3A) and

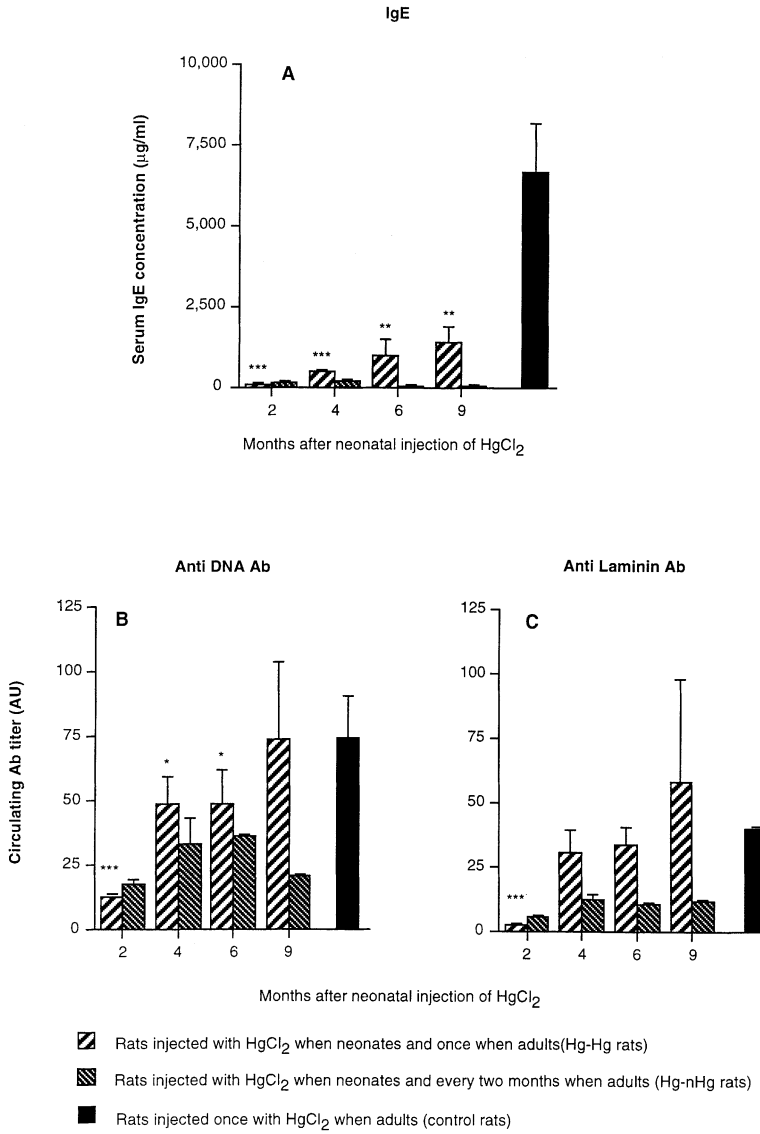

Fig. 3. Neonatally induced tolerance to mercury disease is transient and depends upon the presence of $HgCl_2$. BN rats received a first set of $HgCl_2$ injections when neonates and a second set either at 2, 4, 6 or 9 months of age (Hg-Hg rats, $n = 3$–9) or every 2 months starting at 2 months of age (Hg-nHg rats, $n = 5$). Serum IgE concentration (A) circulating anti-DNA (B) and anti-laminin (C) antibody titers were determined by specific ELISA. Data represent peak values obtained during the last set of $HgCl_2$ injections as compared to data obtained from rats exposed once to $HgCl_2$ at 2 months of age (control rats, $n = 3$). Statistical analysis for Hg-Hg rats versus control rats: $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Statistical analysis for Hg-nHg rats versus Hg-Hg rats exposed again to $HgCl_2$ at 2 months of age: non significant.

circulating anti-DNA (Fig. 3B) antibody titers were lower but gradually increased with time. Moreover, in Hg-Hg rats, circulating anti-laminin antibody titers were significantly lower in rats of 2 months of age, but no significant difference was observed in rats of 4, 6 or 9 months of age as compared to control rats (Fig. 3C). These data indicate that the tolerant state to the mercury disease is transient. However, in rats neonatally injected with $HgCl_2$ and then receiving a second set of $HgCl_2$ injections every 2 months (Hg-nHg rats), the tolerant state to the mercury disease was sustained (Fig. 3A–C).

BN rats neonatally injected with $HgCl_2$ are still susceptible to gold salt-induced immune manifestations

In susceptible BN rats, $HgCl_2$ and gold salts induce similar immune manifestations characterized by polyclonal B cell activation depending upon autoreactive T_h2 cells specific for MHC class II molecules (18,40,,42). To address the specificity of the heavy metal-induced effects, gold salts were administered in BN rats neonatally exposed to $HgCl_2$. As shown in Fig. 4, BN rats neonatally injected with H_2O and exposed at 8–12 weeks of age to ATPS (34) (H_2O-ATPS rats) behaved like unmanipulated BN rats and exhibited an increase in

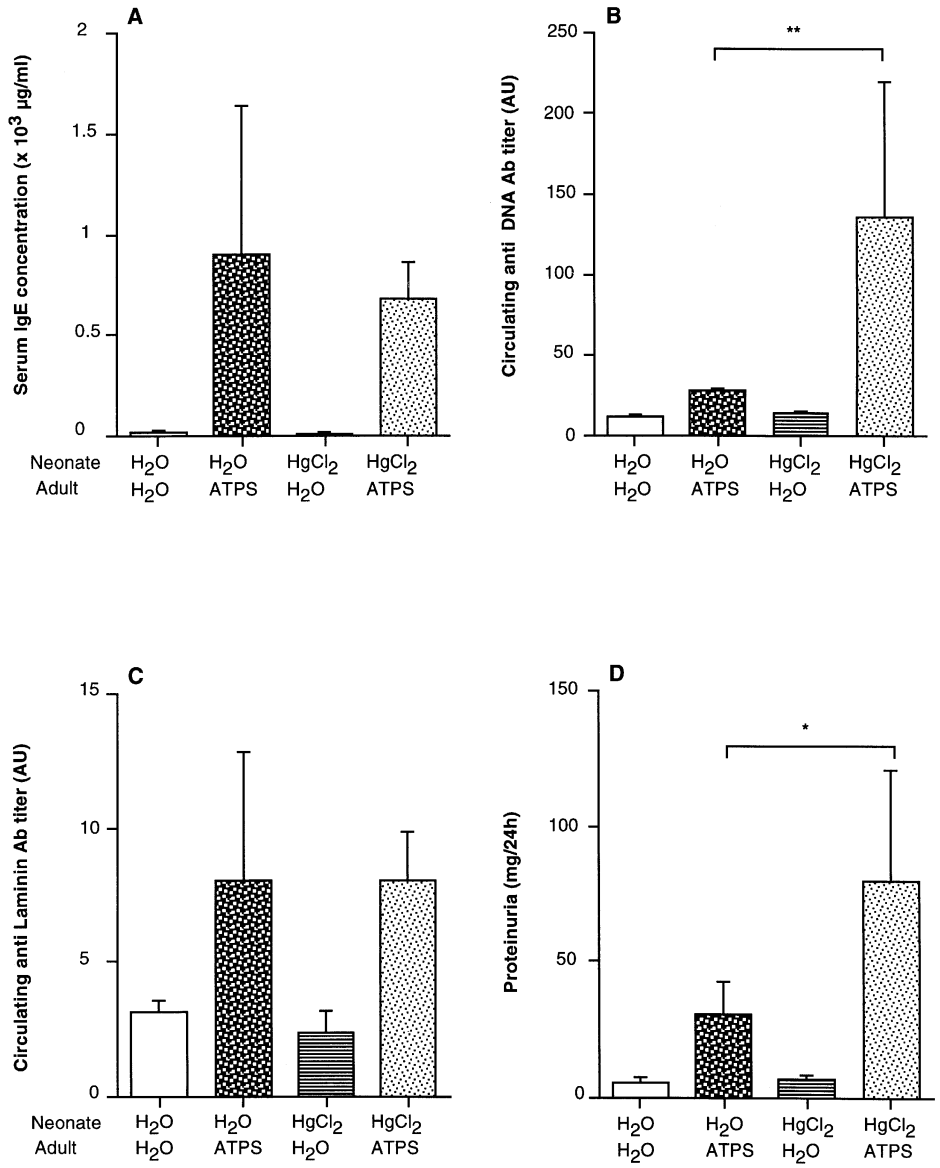

Fig. 4. The tolerance is drug specific. BN rats, when neonates, were injected with H_2O or $HgCl_2$ and then, when 8–12 weeks old (adult), were exposed to H_2O or ATPS (see Methods). Serum IgE concentration (A), circulating anti-DNA (B) and anti-laminin (C) antibody were measured using specific ELISA, and proteinuria (D) was measured using the biuret method. Data represent peak values obtained during the second set of injections, i.e. in adult rats, either on day 14 or 21 of ATPS exposure, and are expressed as the mean ± SD from 5–14 rats. Statistical analysis for $HgCl_2$-ATPS rats versus H_2O-ATPS rats: *$P<0.05$ and **$P<0.01$.

serum IgE level (Fig. 4A), and in circulating antibodies to DNA (Fig. 4B) and to laminin (Fig. 4C). They also demonstrated glomerular linear IgG deposits (Fig. 5a) associated with proteinuria (Fig. 4D). Interestingly, BN rats neonatally injected with $HgCl_2$ and administered with ATPS at 8–12 weeks of age (Hg-ATPS rats) demonstrated ATPS-induced immune manifestations characterized by a closely similar increase in serum IgE concentration (Fig. 4A) and the same titer of anti-laminin antibodies (Fig. 4C) as those of H_2O-ATPS rats. The titers of circulating anti-DNA antibodies were even significantly ($P<0.01$) higher in Hg-ATPS than in H_2O-ATPS rats (Fig. 4B). In the Hg-ATPS rats, glomerular IgG deposits were distributed in the same linear pattern as in H_2O-ATPS rats and associated with granular IgG deposits in arteriolar walls (Fig. 5b); moreover, Hg-ATPS rats developed a proteinuria significantly ($P<0.05$) higher than H_2O-ATPS rats (Fig. 4D).

Tolerance to mercury-induced autoimmunity can be adoptively transferred

Lightly irradiated BN rats, that have received spleen cells originated from naive BN rats and then have been exposed to $HgCl_2$ (control rats), exhibited an increase in serum IgE concentration that peaked at 3180 ± 2600 µg/ml (Fig. 6A), and developed anti-laminin and anti-DNA antibodies whose concentration peaked at 59.3 ± 32.8 and 36.5 ± 17.7 AU respectively (Fig. 6C and B).

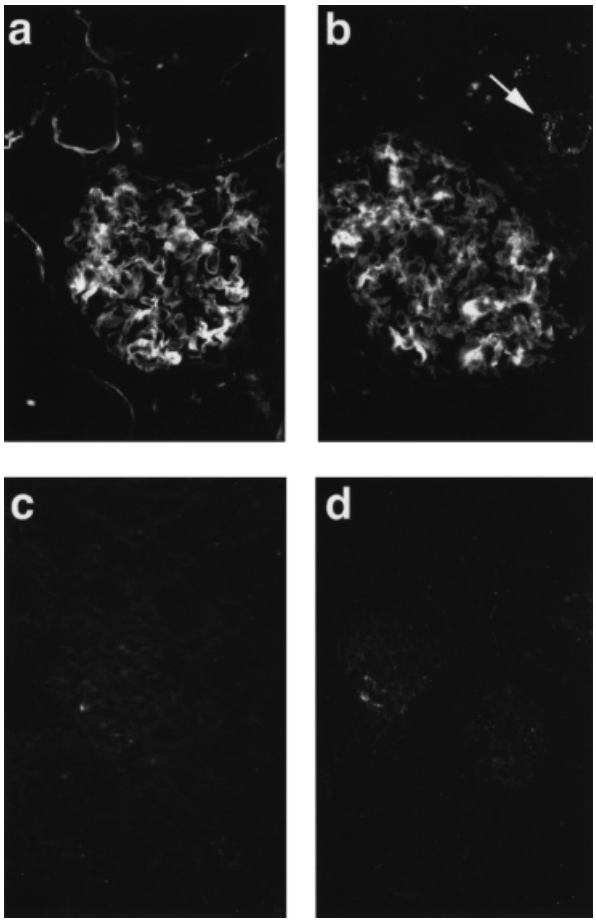

Fig. 5. Immunofluorescence studies. BN rats, when neonates, were injected with H_2O or $HgCl_2$ and then, when 8–12 weeks old (adults), were exposed to H_2O or ATPS (see Methods). Kidney cryostat sections were stained with FITC-labeled sheep anti-rat IgG antibodies. In H_2O-ATPS rats (n = 8) (a; original magnification × 250) linear staining along the glomerular capillary walls is observed; in $HgCl_2$-ATPS rats (n = 5) (b; original magnification ×250). IgG deposits are observed in a linear pattern along the glomerular capillary walls and in a granular pattern in the arteriolar walls (arrow). No staining is ever seen either in H_2O-H_2O rats (n = 14) (c; original magnification ×160) or in Hg-H_2O rats (n = 12) (d; original magnification ×160).

Lightly irradiated BN rats that had received spleen cells originating from BN rats neonatally exposed to $HgCl_2$ and then been exposed to $HgCl_2$ (transfer rats) exhibited, at the peak of production, a significant lower increase in serum IgE concentration as compared to control rats ($P<0.001$) (Fig. 6A). Similarly, the peak of production of anti-laminin antibodies was significantly ($P<0.02$) lowered as compared to control rats (Fig. 6C). Circulating anti-DNA antibody titers were also decreased as compared to control rats, although not significantly (Fig. 6B). In transfer rats, maximal circulating anti-autoantibody titers were never significantly different as compared to BN rats neonatally exposed to $HgCl_2$ and exposed again to $HgCl_2$ after 2 months of age (Hg-Hg rats) (Fig. 6B and C). At the time of sacrifice, i.e. after 8 weeks of $HgCl_2$ exposure, in transfer rats, kidney IgG deposits displayed the same pattern in the mesangial areas as in Hg-Hg rats (not shown); whereas, in control rats, glomerular IgG deposits were distributed in a typical granular pattern along the capillary walls and in the arteriolar walls (not shown).

Discussion

Susceptible BN rats exposed to $HgCl_2$ or ATPS develop a similar T_h2-mediated, systemic autoimmune disease, due to the emergence of autoreactive T_h2 cells that recognize MHC class II molecules. In the present study we demonstrate that: (i) neonatal injections of $HgCl_2$ do not induce the mercury disease in 2-week-old BN neonates and induce immunological tolerance since mercury-induced immunopathological manifestations are abrogated, or profoundly reduced, when these rats are challenged with $HgCl_2$ at 8 weeks of age; (ii) this tolerance is mercury specific because gold-induced immunopathological manifestations are still observed in $HgCl_2$-tolerant rats; (iii) this tolerance is transient but can be sustained providing regular exposure to $HgCl_2$; and (iv) this tolerance is dominant since it is adoptively transferable into syngeneic animals by spleen cells from tolerant rats.

To the best of our knowledge, induction of tolerance to a T_h2-mediated autoimmune model, following neonatal injection of a chemical, has not been previously reported. In mice as well as in rats, we and others demonstrated that injection of F_1 spleen cells into neonates of one parental strain results in transplantation tolerance due to the tolerance of T_h1 cells. In contrast, neonate T_h2 cells that recognize allogeneic MHC class II molecules are present and responsible for B cell polyclonal activation and systemic autoimmunity (23,24,46–48). These results and those from other experimental systems indicate that tolerance is much more difficult to achieve in T_h2 than in T_h1 cells (19,22). In that respect, it is noteworthy that $HgCl_2$ exposure through oral or respiratory routes not only does not induce tolerance, in contrast to what has been observed in several T_h1-mediated autoimmune diseases (13), but leads to systemic autoimmunity (49). However, it has been shown that individuals allergic to bee venom can be desensitized following exposure to tolerogenic amounts of the relevant antigen phospholipase A_2 (50) and the tolerance thus obtained is mediated by IL-10 producing cells that are reminiscent of T_r1 cells (51).

The fine specificity of T cells involved in the $HgCl_2$- and ATPS-induced models of systemic autoimmunity is still unsolved. Our previous data, in both models, indicate that T cells are generated that recognize self-MHC class II molecules or, more likely, a ubiquitous peptide presented in the context of MHC class II molecules (52). Those T cells have a T_h2 phenotype in BN rats, and induce polyclonal B cell activation both *in vivo* and *in vitro* (40,52). In mice exposed to mercury or gold salts, other authors have shown metal-specific T cells but did not evidence their pathogenic role (53). The fact that neonatal injections of $HgCl_2$ induce, in adults, tolerance to $HgCl_2$ but not to ATPS, advocates the existence of such metal-specific T cells. This view is strengthened by our observation that neonatal injections of ATPS induce, in adults, tolerance to ATPS but not to $HgCl_2$ (not shown). Higher anti-laminin antibody titers and proteinuria levels in Hg-ATPS rats than in H_2O-ATPS rats than in H_2O-ATPS rats may be due to a bystander activation of the

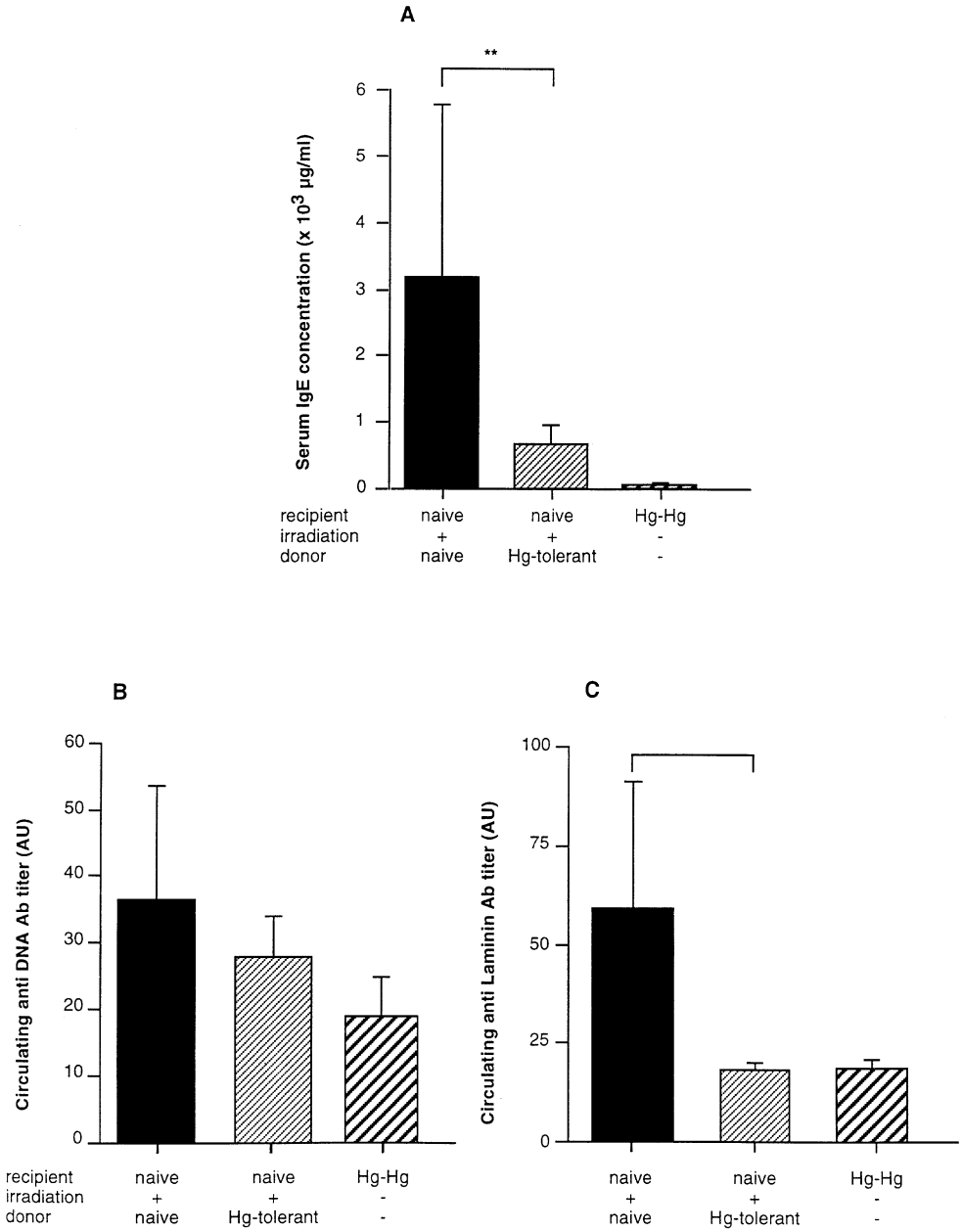

Fig. 6. Tolerance is adoptively transferred by spleen cells. Naive recipient BN rats were ^{137}Cs γ-irradiated (200 rad) and i.v. injected with 100 $\times 10^6$ spleen cells originating from naive BN rats or from BN rats neonatally exposed to $HgCl_2$ (Hg-tolerant rats); transfer of spleen cells was done the day of irradiation; 24 h later, BN recipients and mercury-tolerant littermates (Hg-Hg rats) received injections of 0.5 mg/kg $HgCl_2$ as described in Methods. Serum IgE concentration (A), circulating anti-DNA (B) and anti-laminin (C) antibody titers were determined by specific ELISA. Data represent peak values and are expressed as the mean ± SD from two to six rats. Statistical analysis for recipients of cells from mercury-tolerant rat versus recipients of cells from naive rats: $^*P<0.05$ and $^{**}P<0.01$.

previously suppressed autoreactive T cells specific of the mercury-modified (MHC class II–peptide) complex following the activation of autoreactive T cells primed by the ATPS-modified (MHC class II–peptide) complex. Whether $HgCl_2$ or ATPS is involved in T_h2-mediated autoimmunity, autoreactive T cells that are induced may recognize, in the context of MHC class II molecules, either different ubiquitous peptides or self-peptides specifically altered by the heavy metal (40,54). At this point, one may speculate that whether $HgCl_2$ is administered in neonate or in adult BN rats, T cells of the same specificity are generated but they may differ by their pattern of cytokine production. Those cells, that need to be regularly exposed to $HgCl_2$ in order to maintain their telerogenic potential, are likely to be regulatory cells as demonstrated in other models of tolerance and may produce inhibitory cytokines such as IL-10 or transforming growth factor-β (55–57). This hypothesis is emphasized by the ability of spleen cells from tolerant rats to transfer tolerance in naive

syngeneic recipients. The precise phenotype of these cells, their fine specificity and their profile of cytokine production remain to be investigated. Clonal deletion is another mechanism posited to explain peripheral tolerance (5). In previous experiments, we demonstrated that in adult BN rats, $HgCl_2$ induces a polyclonal T cell expansion (58) and in BN rats neonatally injected with $HgCl_2$ no changes occur in the T cell repertoire (not shown). Considering these findings clonal deletion might be ruled out as an associated mechanism of tolerance to the mercury disease. Taken together our data favor specific dominant tolerance due to regulatory cells rather than to clonal deletion; however, anergy as an associated mechanism of tolerance cannot be ruled out.

We have previously shown that, besides the induction of autoreactive T cells, $HgCl_2$ induces *in vitro* IL-4 gene expression in normal T cells following a protein kinase C-dependent pathway (59,60). This latter effect is still observed in tolerant animals (not shown) and likely to explain IgE production that, if deeply reduced, is not completely abrogated and furthermore plateaued after 2 weeks of $HgCl_2$ administration. Thus our data indicate that production of IL-4 associated with the direct effect of $HgCl_2$ on the encoding gene is not affected by neonatal exposure to $HgCl_2$ and nevertheless not sufficient to induce the mercury disease. This suggests a very efficient regulatory process, even more potent than towards the production of autoantibodies. $CD8^+$ T cells that have been involved in IgE tolerance (61) may be at play. Moreover, previous studies have shown that $HgCl_2$ exposure not only induces autoimmune manifestations that spontaneously resolve even if $HgCl_2$ injections are continued, but also makes the animals resistant to rechallenge with full-dose $HgCl_2$ (36). The exact mechanisms involved in regulation and resistance are still ill-defined. There is some evidence that T_h1/T_h2 balance may be involved (42,43,62,63). Indeed the importance of T_h1 cells has been suggested since anti-IL-2 receptor antibody treatment, that preferentially blocks the effect of T_h1 cells, delays the regulatory phase (42). It has been shown that depletion of $CD8^+$ T cells partially reverses the resistant state without affecting the regulatory phase (36). Furthermore, adoptive transfer of $CD8^+$ T cells from resistant rats can transfer resistance in naive syngeneic rats (35,36). In the present study, we show that neonatal administration of $HgCl_2$ is not pathogenic and induces the development of disease resistance in adult animals. This neonatally induced resistant state is specific and can be transferred to naive recipients with spleen cells, indicating a role of dominant tolerance. Co-transfer of spleen cells from naive and mercury-tolerant animals remains to be investigated to emphasize this phenomenon of active suppression. Whether this state of neonatally-induced resistance is similar to that described in adult rats treated with $HgCl_2$ remains to be determined; particularly, whether $CD4^+$ or $CD8^+$ T cells are involved in the transfer and maintenance of neonatal tolerance remains to be determined.

In summary, our findings of a solid tolerant state induced by neonatal injections of $HgCl_2$ indicate that neonatal tolerance can be induced to a systemic autoimmune disease mediated by T_h2 cells. Further investigation of this dominant tolerance will be of major interest because it might be instrumental in lupus autoimmunity and allergy.

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Abbreviations

ATPS	sodium aurothiopropanolsulfonate
BN	Brown-Norway
EAE	experimental autoimmune encephalomyelitis
HRP	horseradish peroxidase

References

1 Billingham, R. E., Brent, L. and Medawar, P. B. 1953. Actively acquired tolerance of foreign cells. *Nature* 172:603.
2 Sarzotti, M., Robbins, D. S. and Hoffman, P. M. 1996. Induction of protective CTL responses in newborn mice by murine retrovirus. *Science* 271:1726.
3 Ridge, J. P., Fuchs, E. J. and Matzinger, P. 1996. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* 271:1723.
4 Forsthuber, T., Yip, H. C. and Lehmann, P. V. 1996. Induction of TH1 and TH2 immunity in neonatal mice. *Science* 271:1728.
5 Cobbold, S. P., Adams, E., Marshall, S. E. Davies, J. D. and Waldmann, H. 1996. Mechanisms of peripheral tolerance and suppression induced by monoclonal antibodies to CD4 and CD8. *Immunol. Rev.* 149:5.
6 Saoudi, A., Seddon, B., Heath, V., Fowell, D. and Mason, D. 1996. The physiological role of regulatory T cells in the prevention of autoimmunity: the function of the thymus in the generation of the regulatory T cell subset. *Immunol. Rev.* 149:195.
7 Khoruts, A., Miller, S. D. and Jenkins, M. K. 1995. Neuroantigen-specific T_h2 cells are inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector T_h1 cells. *J. Immunol.* 155:5011.
8 Balomenos, D., Rumold, R. and Theofilopoulos, A. N. 1998. Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-*lpr* mice. *J. Clin. Invest.* 101:364.
9 Takahashi, S., Fossati, L., Iwamoto, M., Merino, R., Motta, R., Kobayakawa, T. and Izui S. 1996. Imbalance towards T_h1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. *J. Clin. Invest.* 97:1597.
10 Koevary, S. B. and Caspi, R. R. 1997. Prevention of experimental autoimmune uveoretinitis by intrathymic S-antigen injection. *Ocul. Immunol. Inflamm.* 5:165.
11 Wilson, D. B., Wilson, D. H. and Schroder, K. 1998. Acquired thymic tolerance and experimental allergic encephalomyelitis in the rat. I. Parameters and analysis of possible mechanisms. *Eur. J. Immunol.* 28:2770.
12 Chen, W., Sayegh, M. H. and Khoury, S. J. 1998. Mechanisms of acquired thymic tolerance *in vivo*: intrathymic injection of antigen induces apoptosis of thymocytes and peripheral T cell anergy. *J. Immunol.* 160:1504.
13 Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A. and Weiner, H. L. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelotis. *Science* 265:1237.
14 Kumar, V. and Sercarz, E. E. 1993. The involvement of T cell receptor peptide-specific regulatory $CD4^+$ T cells in recovery from antigen-induced autoimmune disease. *J. Exp. Med.* 178:909.
15 Lafaille, J. J., Keere, F. V., Hsu, A. L., Baron, J. L., Haas, W., Raine, C. S. and Tonegawa, S. 1997. Myelin basic protein-specific T helper 2 (T_h2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J. Exp. Med.* 186:307.

16 Singh, R. R., Hahn, B. H. and Secarz, E. E. 1996. Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: implications for antibody vs. T cell-mediated autoimmunity. *J. Exp. Med.* 183:1613.
17 Nakajima, A., Hirose, S., Yagita, H. and Okumura, K. 1997. Roles of IL-4 and IL-12 in the development of lupus in NZB/W F_1 mice. *J. Immunol.* 158:1466.
18 Goldman, M., Druet, P. and Gleichman, E. 1991. T_h2 cells in systemic autoimmunity: insights from allogeneic diseases and chemically-induced autoimmunity. *Immunol. Today* 12:223.
19 Gilbert, K. M., Hoang, K. D. and Weigle, W. O. 1990. T_h1 and T_h2 clones differ in their response to a telerogenic signal. *J. Immunol.* 144:2063.
20 De Witt, D., Van Mechelen, M., Ryelandt, M., Figuieredo, A. C., Abramowicz, D., Goldman, M., Bazin, H., Urbain, J. and Leo, O. 1992. The injection of deaggregated gamma globulins in adult mice induces antigen-specific unresponsiveness of T helper type 1 but not type 2 lymphocytes. *J. Exp. Med.* 175:9.
21 Sloan-Lancaster, J., Evavold, B. D. and Allen, P. M. 1994. T_h2 cell clonal anergy as a consequence of partial activation. *J. Exp. Med.* 180:1195.
22 Romball, C. G. and Weigle, W. O. 1993. *In vivo* induction of tolerance in murine $CD4^+$ cell subsets. *J. Exp. Med.* 178:1637.
23 Schurmans, S., Heusser, C. H., Qin, H.-Y., Merino, J., Brighouse, G. and Lambert, P. H. 1990. *In vivo* effects of anti-IL-4 monoclonal antibody on neonatal induction of tolerance and on an associated autoimmune syndrome. *J. Immunol.* 145:2465.
24 Abramowicz, D., Durez, P., Gerard, C., Donckier, V., Amraoui, Z., Velu, T. and Goldman, M. 1993. Tolerance of vascularized allografts and mechanisms. Neonatal induction of transplantation tolerance in mice is associated with *in vivo* expression of IL-4 and -10 mRNAs. *Transplant. Proc.* 25:312.
25 Pelletier, L., Pasquier, R., Guettier, C., Vial, M. C., Mandet, C., Nochy, D., Bazin, H. and Druet, P. 1998. $HgCl_2$ induces T and B cells to proliferate and differentiate in BN rats. *Clin. Exp. Immunol.* 71:336.
26 Hirsch, F., Couderc, J., Sapin, C., Fournie, G. and Druet, P. 1982. Polyclonal effect of $HgCl_2$ in the rat, its possible role in an experimental autoimmune disease. *Eur. J. Immunol.* 12:620.
27 Aten, J., Bosman, C. B., Rozing, J., Stijnen, T., Hoedemaeker, P. J. and Weening, J. J. 1988. Mercuric chloride-induced autoimmunity in the Brown Norway rat. Cellular kinetics and major histocompatibility complex antigen expression. *Am. J. Pathol.* 133:127.
28 Prouvost-Danon, A., Abadie, A., Sapin, C., Bazin, H. and Druet, P. 1981. Induction of IgE synthesis and potentiation of anti-ovalbumin IgE response by $HgCl_2$ in the rat. *J. Immunol.* 126:699.
29 Bellon, B., Capron, M., Druet, E., Verroust, P., Vial, M. C., Sapin, C., Girard, J. F., Foidart, J. M., Mahieu, P. and Druet, P. 1982. Mercuric chloride induced autoimmune disease in Brown-Norway rats. Sequential search for anti-basement membrane antibodies and circulating immune complexes. *Eur. J. Clin. Invest.* 12:127.
30 Fukatsu, A., Brentjens, J. R., Killen, P. D., Kleinman, H. K., Martin, G. R. and Andres, G. A. 1987. Studies on the formation of glomerular immune deposits in Brown-Norway rats injected with mercuric chloride. *Clin. Immunol. Immunopathol.* 45:35.
31 Pusey, C. D., Bowman, C., Morgan, A., Weetman, A. P., Hartley, B. and Lockwood, C. M. 1990. Kinetics and pathogenicity of autoantibodies induced by mercuric chloride in the Brown-Norway rat. *Clin. Exp. Immunol.* 81:76.
32 Sapin, C., Druet, E. and Druet, E. and Druet, P. 1977. Induction of anti-glomerular basement membrane antibodies in the Brown-Norway rat by mercuric chloride. *Clin. Exp. Immunol.* 28:173.
33 Druet, P., Druet, E., Potdevin, F. and Sapin, C. 1978. Immune type glomerulonephritis induced by $HgCl_2$ in the Brown Norway rat. *Ann. Immunol.* 129:777.
34 Tournade, H., Pelletier, L., Guéry, J. C., Pasquier, R., Nochy, D., Hinglais, N., Guilbert, B. and Druet, P. 1991. Experimental gold-induced autoimmunity. *Nephrol. Dial. Transplant.* 6:621.
35 Bowman, C., Mason, D. W., Pusey, C. D. and Lockwood, C. M. 1984. Autoregulation of antibody synthesis in mercuric chloride nephritis in the Brown-Norway rat. I. A role for T suppressor cells. *Eur. J. Immunol.* 14:464.
36 Mathieson, P. W., Stapleton, K. J., Oliveira, D. B., and Lockwood, C. M. 1991. Immunoregulation of mercuric chloride-induced autoimmunity in Brown-Norway rats: a role for $CD8^+$ T cells revealed by *in vivo* depletion studies. *Eur. J Immunol* 21:2105.
37 Fillastre, J. P., Druet, P. and Merry, J. P. 1988. Proteinuric nephropathies associated with drugs and substances of abuse. In Cameron, J. S. and Glassock, R. J., eds, *The Nephrotic Syndrome*, p. 697. Basel, New York.
38 Rossert, J., Pelletier, L., Pasquier, R. and Druet, P. 1988. Autoreactive T cells in mercury-induced autoimmunity. Demonstration by limiting dilution analysis. *Eur. J. Immunol.* 18:1761.
39 Pelletier, L., Pasquier, R., Rossert, J., Vial, M. C., Mandet, C. and Druet, P. 1988. Autoreactive T cells in mercury-induced autoimmunity. Ability to induce the autoimmune disease. *J. Immunol.* 140:750.
40 Saoudi, A., Castedo, M., Nochy, D., Mandet, C., Pasquier, R., Druet, P. and Pelletier, L. 1995. Self reactive anti-class II T_h2 cell lines derived from gold salt-injected rats trigger B cell polyclonal activation and transfer autoimmunity in CD8-depleted normal syngeneic recipients. *Eur. J. Immunol.* 25:1972.
41 Ochel, M., Vohr, H. W., Pfeiffer, C. and Gleichmann, E. 1991. IL-4 is required for the IgE and IgG1 autoantibody formation in mice treated with mercuric chloride. *J. Immunol.* 146:3006.
42 Dubey, C., Kuhn, J., Vial, M. C., Druet, P. and Bellon, B. 1993. Anti-interleukin-2 receptor monoclonal antibody therapy supports a role for T_h1-like cells in $HgCl_2$-induced autoimmunity in rats. *Scand. J. Immunol.* 37:406.
43 Gillespie, K. M., Sauodie, A., Kuhn, J., Whittle, C. J., Druet, P., Bellon, B. and Mathieson, P. W. 1996. T_h1/T_h2 cytokine gene expression after mercuric chloride in susceptible and resistant rat stains. *Eur. J. Immunol.* 26:2388.
44 Guéry, J. C., Druet, E., Glotz, D., Hirsch, F., Mandet, C., De Heer, E. and Druet, P. 1990. Specificity and cross-reactive idiotypes of anti-glomerular basement membrane autoantibodies in $HgCl_2$-induced autoimmune glomerulonephritis. *Eur. J. Immunol.* 20:93.
45 Aarden, L. A. and Smeenk, R. J. T. 1981. In Lefkovits, I. and Pernes, B., eds., *Measurement of Antibodies Specific for DNA*, vol. 2, p. 75. Academic Press, New York.
46 Chen, N. and Field, E. H. 1995. Enhanced type 2 and diminished type 1 cytokines in neonatal tolerance. *Transplantation* 59:933.
47 Donckier, V., Wissing, M., Bruyns, C., Abramowicz, D., Lybin, M., Vanderhaeghen, M.-L. and Goldman, M. 1995. Critical role of interleukin 4 in the induction of neonatal transplantation. *Transplantation* 59:1571.
48 Dubey, C., Kuhn, J., Wissing, M., Nisol, F., Chavez, M., Bazin, H., Goldman, M., Druet, P. and Bellon, B. 1992. Susceptibility and resistance to autoimmunity following neonatal injection of semi-allogeneic spleen cells in rats. *J. Autoimm.* 5:629.
49 Bernaudin, J. F., Druet, E., Druet, P. and Masse, R. 1981. Inhalation or ingestion of organic or inorganic mercurials produces auto-immune disease in rats. *Clin. Immunol. Immunopathol.* 20:129.
50 Akdis, C. A., Blesken, T., Akdis, M., Wuthrich, B. and Blaser, K. 1998. Role of interleukin 10 in specific immunotherapy. *J. Clin. Invest.* 102:98.
51 Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. and Roncarolo, M. G. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737.
52 Druet, P. and Pelletier, L. 1996. Th_1 and T_h2 autoreactive anti-class II cell lines in the rat suppress or induce autoimmunity. *J. Autoimmun.* 9:221.
53 Kubicka-Muranyi, M., Griem, P., Lübben, B., Rottman, N., Lührmann, R., Beyer, K. and Gleichmann, E. 1995. Mercuric chloride-induced autoimmunity in mice involves an up-regulation presentation of altered and unaltered nucleolar self antigen. *Int. Arch. Allergy Immunol.* 108:1.
54 Griem, P. and Gleichmann, E. 1995. Metal ion-induced autoimmunity. *Curr. Opin. Immunol.* 7:831.
55 Miller, A., Lider, O., Roberts, A. B., Sporn, M. B. and Weiner, H. L. 1992. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both *in vitro* and *in vivo* immune

responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc. Natl Acad. Sci. USA* 89:421.
56 Kosiewicz, M. M. and Streilein, J. W. 1996. Intraocular injection of class II-restricted peptide induces an unexpected population of CD8 regulatory cells. *J. Immunol.* 157:1905.
57 Seddon, B. and Mason, D. 1999. Regulatory T cells in the control of autoimmunity: the essential role of transforming growth factor beta and interleukin 4 in the prevention of autoimmune thyroiditis in rats by peripheral CD4(+)CD45RC(–) cells and CD4(+)CD8(–) thymocytes. *J. Exp. Med.* 189:279.
58 Fillion, J., Baccala, R., Pannetier, C., Kuhn, J., Druet, P. and Bellon, B. 1997. Evidence for heterogeneous TCR V beta repertoire expression in mercury-induced immune disorders in rats. *Int. Immunol.* 9:263.
59 Prigent, P., Saoudi, A., Pannetier, C., Graber, P., Bonnefoy, J. Y., Druet, P. and Hirsch, F. 1995. Mercuric chloride a chemical responsible for T helper cell (T_h)2-mediated autoimmunity in Brown-Norway rats directly triggers T cells to produce Interleukin-4. *J. Clin. Invest.* 96:1484.
60 Badou, A., Savignac, M., Moreau, M., Leclerc, C., Pasquier, R., Druet, P. and Pelletier, L. 1997. $HgCl_2$-induced interleukin-4 gene expression in T cells involves a protein kinase C-dependent calcium influx through L-type calcium channels. *J. Biol. Chem.* 272:32411.
61 Chen, S. S. 1991. Mechanisms of IgE tolerance: dual regulatory T cell lesions in perinatal IgE tolerance. *Eur. J. Immunol.* 21:2461.
62 Castedo, M., Pelletier, L., Rossert, J., Pasquier, R., Villarroya, H. and Druet, P. 1993. Mercury-induced autoreactive anti-class II T cell line protects from experimental autoimmune encephalomyelitis by the bias of $CD8^+$ antiergotypic cells in Lewis rats. *J. Exp. Med.* 177:881.
63 Szeto, C., Gillespie, K. M. and Mathieson, P. W. 1999. Low-dose mercuric chloride induces resistance in brown norway rats to further mercuric chloride by up-regulation of interferon-gamma. *Scand. J. Immunol.* 50:195.