Neonatal induction of tolerance to Th2-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 (HgCl2) or sodium aurothiopropanolsulfonate (ATPS), they develop a syndrome characterized by a polyclonal B cell activation depending upon CD4+ Th2 cells that recognize self-MHC class II molecules. Since peripheral tolerance of Th2 cells might be crucial in the prevention of immunological manifestations such as allergy, establishing conditions for inducing tolerance to HgCl2- or ATPS-mediated immune manifestations appeared to be of large interest. We report here that BN rats neonatally injected with HgCl2: (i) do not develop the mercury disease, (ii) remain resistant to HgCl2-induced autoimmunity at 8 weeks of age and later, provided they are regularly exposed to HgCl2, (iii) are still susceptible to ATPS-induced immune manifestations, and (iv) exhibit spleen cells that adoptively transfer tolerance to HgCl2-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 Th2-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 Tn1 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, Th2 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, Th2 cells were first accepted as not susceptible to tolerization (19,20) and even though induction of peripheral tolerance of Th2 cells is no longer controversial (21), a tolerant state appears more difficult to achieve in Th2 than in Th1 cells (22). For example, injections of parental spleen cells in F1 neonates lead to tolerance of Th1 cells, whereas Th2 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 Th2-mediated autoimmunity. Indeed BN rats injected with mercuric chloride (HgCl2) or sodium aurothiopropanolsulfonate (ATPS) develop a similar lupus-like syndrome with lymphoproliferation (25–27), hypergammaglobulinemia affecting mainly IgE and IgG1 (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 HgCl2.
BN rats were neonatally injected with HgCl$_2$ (Hg rats) or H$_2$O (H$_2$O-H$_2$O rats) or ATPS (Hg-ATPS and H$_2$O-ATPS rats) (second set of injections). In another set, rats were re-exposed to HgCl$_2$ either at 2, 4, 6 or 9 months of age. sera containing known amounts of rat IgE.

**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$_2$O 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$_2$O (H$_2$O 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$_2$O-Hg rats) or H$_2$O (Hg-H$_2$O or H$_2$O-H$_2$O rats) or ATPS (Hg-ATPS or H$_2$O-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$_2$O were i.v. transferred into $^{137}$Cs γ-irradiated (200 rad) BN rats of 8–12 weeks of age.

**Comparisons with values of maximum binding activity of serum IgE concentration**

**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 serum reference.

**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).

**Quantification of serum IgE concentration**

Individual serum IgE concentrations were determined by a sandwich ELISA as follows. Microtiter plates (Maxi-Sorp; Nunc, Rockside, 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.

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**Table 1. Effect of HgCl$_2$ administration in BN neonates**

<table>
<thead>
<tr>
<th>Neontal injections</th>
<th>$n$</th>
<th>IgE concentration (µg/ml)</th>
<th>Anti-laminin antibody titer (AU)</th>
<th>Glomerular Ig deposits</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>9</td>
<td>$1.4 \pm 0.6$</td>
<td>$1.3 \pm 0.1$</td>
<td>–</td>
</tr>
<tr>
<td>HgCl$_2$</td>
<td>9</td>
<td>$2.3 \pm 0.5$</td>
<td>$1.6 \pm 0.1^c$</td>
<td>–</td>
</tr>
</tbody>
</table>

*Neonates received six injections of H$_2$O or HgCl$_2$ starting within 24 h after birth and were sacrificed at 2 weeks of age.  
*Expressed as percent of maximum binding activity of a serum reference.  
*Not significant using the Student’s t-test when compared to H$_2$O-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).
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Fig. 1. BN rats neonatally injected with HgCl₂ are resistant to mercury disease under HgCl₂ exposure at 8–12 weeks of age. BN rats, when neonates, were injected with H₂O or HgCl₂ and then, when 8–12 weeks old (adults), were exposed to H₂O or HgCl₂ (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 H₂O-Hg rats: **P<0.01 and ***P<0.001.

Results

Neonatal injections of HgCl₂ make BN rats tolerant to HgCl₂-induced autoimmunity

Neonatally HgCl₂-injected BN rats sacrificed at 2 weeks of age, i.e., after six injections of HgCl₂, exhibited similar very low levels of circulating IgE and antibodies to laminin as neonatally H₂O-injected BN rats sacrificed at 2 weeks of age. Moreover, in both groups renal glomeruli were free of IgG deposits (Table I).

Neonatally H₂O-injected BN rats exposed to HgCl₂ at 8–12 weeks of age (H₂O-Hg rats) exhibited the typical HgCl₂-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 HgCl₂ administration (not shown). Moreover, on day 15 all these H₂O-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 HgCl₂ 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 H₂O-Hg rats developed proteinuria (Fig. 1D).

In sharp contrast, neonatally HgCl₂-injected BN rats exposed to HgCl₂ 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 H₂O or HgCl₂ and then, when 8–12 weeks old (adults), were exposed to H₂O or HgCl₂ (see Methods). Kidney cryostat sections were stained with FITC-labeled sheep anti-rat IgG antibodies. In H₂O-Hg rats (n = 16), on day 15 of HgCl₂ exposure (a; original magnification ×250), IgG deposits are observed along the glomerular capillary walls in a linear pattern, and on day 60 of HgCl₂ 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 HgCl₂ 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 H₂O-H₂O rats (n = 14) (2; original magnification ×160) or in Hg-H₂O rats (n = 12) (f; original magnification ×160).
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Fig. 3. Neonatally induced tolerance to mercury disease is transient and depends upon the presence of HgCl₂. BN rats received a first set of HgCl₂ 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 HgCl₂ injections as compared to data obtained from rats exposed once to HgCl₂ 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 HgCl₂ 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 HgCl₂ and then receiving a second set of HgCl₂ injections every 2 months (Hg-nHg rats), the tolerant state to the mercury disease was sustained (Fig. 3A–C).

BN rats neonatally injected with HgCl₂ are still susceptible to gold salt-induced immune manifestations

In susceptible BN rats, HgCl₂ and gold salts induce similar immune manifestations characterized by polyclonal B cell activation depending upon autoreactive Th2 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 HgCl₂. As shown in Fig. 4, BN rats neonatally injected with H₂O and exposed at 8–12 weeks of age to ATPS (34) (H₂O-ATPS rats) behaved like unmanipulated BN rats and exhibited an increase in
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Fig. 4. The tolerance is drug specific. BN rats, when neonates, were injected with H2O or HgCl2 and then, when 8–12 weeks old (adult), were exposed to H2O 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 HgCl2-ATPS rats versus H2O-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 HgCl2 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 H2O-ATPS rats. The titers of circulating anti-DNA antibodies were even significantly (P<0.01) higher in Hg-ATPS than in H2O-ATPS rats (Fig. 4B). In the Hg-ATPS rats, glomerular IgG deposits were distributed in the same linear pattern as in H2O-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 H2O-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 HgCl2 (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).
compared to BN rats neonatally exposed to HgCl₂ and existence of such metal-speci-
cific T cells (Fig. 6B and C). At the time of sacri-
fice, maximal circulating anti-DNA antibody titers were also to mercury or gold salts, other authors have shown metal-
induced immunopathological manifesta-
tions are still observed in HgCl₂-tolerant rats; (iii) this tolerance is transient but can be
sustained providing regular exposure to HgCl₂; 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
Th₂-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
F₁ spleen cells into neonates of one parental strain results in
transplantation tolerance due to the tolerance of T₁ cells. In
contrast, neonate T₁ cells that recognize allogeneic MHC
class II molecules are present and responsible for B cell
disensitized following exposure to tolerogenic amounts of
the relevant antigen phospholipase A₂ (50) and the tolerance
thus obtained is mediated by IL-10 producing cells that are
reminiscent of Tr₁ cells (51).

The fine specificity of T cells involved in the HgCl₂-
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 molec-
ules or, more likely, a ubiquitous peptide presented in the
context of MHC class II molecules (52). Those T cells have a
Th₂ 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 HgCl₂ induce, in
adults, tolerance to HgCl₂ 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 HgCl₂
(not shown). Higher anti-laminin antibody titers and proteinuria
levels in Hg-ATPS rats than in H2O-ATPS rats than in H2O-
ATPS rats may be due to a bystander activation of the

Discussion

Susceptible BN rats exposed to HgCl₂ or ATPS develop a
similar T₁2-mediated, systemic autoimmune disease, due to
the emergence of autoreactive T₁2 cells that recognize MHC
class II molecules. In the present study we demonstrate that:
(i) neonatal injections of HgCl₂ do not induce the mercury
disease in 2-week-old BN neonates and induce immunological
tolerance since mercury-induced immunopathological mani-
festations are abrogated, or profoundly reduced, when these
rats are challenged with HgCl₂ at 8 weeks of age; (ii) this
tolerance is mercury specific because gold-induced
immunopathological manifestations are still observed in
HgCl₂-tolerant rats; (iii) this tolerance is transient but can be
sustained providing regular exposure to HgCl₂; and (iv) this
tolerance is dominant since it is adoptively transferable into
syngeneic animals by spleen cells from tolerant rats.

Lightly irradiated BN rats that had received spleen cells
originating from BN rats neonatally exposed to HgCl₂ and
then been exposed to HgCl₂ (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 HgCl₂ and
exposed again to HgCl₂ after 2 months of age (Hg-Hg rats)
(Fig. 6B and C). At the time of sacrifice, i.e. after 8 weeks
of HgCl₂ exposure, in transfer rats, kidney IgG deposits dis-
played 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).
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Fig. 6. Tolerance is adoptively transferred by spleen cells. Naive recipient BN rats were $^{137}$Cs $\gamma$-irradiated (200 rad) and i.v. injected with $10^6$ 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 telorogenic 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-$\beta$ (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 postulated to explain peripheral tolerance (5). In previous experiments, we demonstrated that in adult BN rats, HgCl₂ induces a polyclonal T cell expansion (58) and in BN rats neonatally injected with HgCl₂ 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, HgCl₂ 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 1 Billingham, R. E., Brent, L. and Medawar, P. B. 1953. Actively

pathway (59,60). This latter effect is still observed in tolerant

sion in normal T cells following a protein kinase C-dependent

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9 Takahashi, S., Fossati, L., Iwamoto, M., Merino, R., Motta, R., Kobayakawa, T. and Izui S. 1996. Imbalance towards T h1 state without affecting the regulatory phase (36). Furthermore, adoptive transfer of CD8+ T cells from resistant rats can transfer resistance in naïve syngeneic rats (35,36). In the present study, we show that neonatal administration of HgCl₂ 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 HgCl₂ 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 HgCl₂ indicate that neonatal tolerance can be induced to a systemic autoimmune disease mediated by T₁₂ 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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responses by the release of transforming growth factor beta after
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further mercuric chloride by up-regulation of interferon-gamma.