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IL-12 Down-Regulates Autoantibody Production in Mercury-Induced Autoimmunity

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In genetically susceptible H-2 haplotype mice, subtoxic doses of mercuric chloride (HgCl₂) induce a complex autoimmune syndrome characterized by the production of non-nuclear IgG Abs, lymphoproliferation, increased serum levels of IgG1 and IgE Abs, and renal Ig deposits. Mercury-induced autoimmunity in H-2 haplotype mice provides a useful model for chemically related autoimmunity in humans. The increase in serum IgG1 and IgE, which are under IL-4 control, suggests a role for the Th2 subset in this syndrome. The IL-12 cytokine induces T cell proliferation and IFN-γ production and is necessary for differentiation of naive T cells into the Th1 subset. To gain an understanding of T cell control in this syndrome and, in particular, Th1/Th2 regulation, we assessed the effect of IL-12 administration in mercury-induced autoimmunity. Groups of A.SW mice (H-2 haplotype) received HgCl₂ plus IL-12, HgCl₂ alone, or IL-12 alone. IL-12 treatment resulted in a dramatic reduction of the anti-nuclear Ab titers. IL-12 also inhibited the HgCl₂-induced serum IgG1 increase, but, in contrast, did not significantly affect IgE induction in this model. This observation may be related to our unexpected finding that IL-12 further potentiated the HgCl₂-triggered IL-4 induction in this model. The levels of renal Ig deposits were similar in mice receiving HgCl₂ alone or HgCl₂ plus IL-12. Our results indicate that IL-12 can down-regulate the autoimmune component of this experimental syndrome and that the various manifestations of mercury-induced autoimmunity are independently regulated. The Journal of Immunology, 1998, 160: 1612–1617.

Mice

Mouse and human Th cells consist of two subgroups, Th1 and Th2, characterized by distinct cytokine secretion patterns (1, 2). Th1 cells secrete IL-2 and IFN-γ, while Th2 cells secrete IL-4, IL-5, and IL-10. The development of Th1 and Th2 subsets from naive precursor cells depends upon cytokines present during the initiation of the immune response. IL-12 is critical for the development of the Th1 phenotype and thereby for initiating an inflammatory immune response (3). As a corollary to the Th1-promoting role of IL-12, Th2-mediated diseases can be prevented by the administration of IL-12. In a murine model of leishmaniasis, susceptible BALB/c mice are protected from Th2-mediated disease by IL-12 administration at the time of parasite challenge (4, 5). Conversely, the presence of IL-12 is a contributor to certain Th1-mediated autoimmune diseases, and Abs to the p40 component of the IL-12 molecule can abrogate disease (6). A targeted deletion of the gene coding for the IL-12 p40 subunit results in a defect in generating a Th1 response and prevents diabetes development in NOD mice (7). Thus, IL-12 serves as a potentially important immunoregulator in determining disease outcome.

Mouse or rat strains expressing certain MHC Ags are exquisitely susceptible to the heavy metal induction of a complex autoimmune syndrome. In susceptible H-2 haplotype mice, subtoxic doses of mercuric chloride (HgCl₂) induce an autoimmune dysfunction characterized by the production of anti-nuclear autoantibodies (ANoAs) (8), lymphoproliferation, hyperglobulinemia (especially pronounced for IgG1 and IgE), and mild glomerulonephritis with renal Ig deposits. These manifestations peak 2 to 3 wk after the beginning of the Hg injections, and the ANoAs can persist for as long as 1 yr after the induction phase. Most of the other manifestations, however, spontaneously resolve in about 4 to 5 wk, even if HgCl₂ injections are continued. The increase in serum IgG1 and IgE in Hg-treated mice suggests that Th2 cells play an important role in the pathogenesis of this syndrome (10, 12). HgCl₂ can directly induce IL-4 secretion in susceptible strains (13–15), and some of the manifestations can be prevented by anti-IL-4 treatment (16). The role of Th1 cells in this syndrome is less well defined, although it has been suggested that this subset might be involved in the regulation phase of the disease. In the rat, depletion of the Th1-like subset (OX22(high)) leads to an exacerbation of tissue injury, presumably due to a loss of Th1 regulation (17). In mice, treatment with anti-CD2 Abs, which is believed to down-regulate the Th1 pathway, exacerbates the manifestations of mercury-induced autoimmunity (18).

We reasoned that administration of IL-12, a potent Th1 inducer, may be able to bypass the Th2 phase of the disease and result in early induction of the regulatory Th1 cells. In the present study we examined the effect of IL-12 administration on the development of Hg-induced autoimmunity in A.SW (H-2 haplotype) mice. Our results indicate that IL-12 can dramatically decrease the autoantibody component of the syndrome, but that it has differing effects on the other manifestations of the syndrome.

Materials and Methods

Mice

Female A.SW/SnJ (H-2 haplotype) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in our animal facilities.

3 Abbreviations used in this paper: ANoAs, anti-nuclear Abs; PT, PBS and 0.05% Tween; AP, alkaline phosphatase; PBTN, PBS containing 1% BSA, 0.05% Tween-20, and 0.02% sodium azide; HBsAg, hepatitis B e Ag; GVHD, graft-vs-host disease.
HgCl₂ and IL-12 treatment

Groups of at least 2-mo-old mice were injected with HgCl₂ alone, IL-12 alone, or both HgCl₂ and IL-12 as follows. Mice treated with HgCl₂ were injected three times a week s.c. with 30 μg of HgCl₂ (Sigma Chemical Co., St. Louis, MO) in 100 μl of sterile PBS throughout the duration of the experiment (16). Mice treated with rIL-12 (gift from Genetics Institute, Inc., Cambridge MA) received a standard regimen of four injections of 0.2 μg of IL-12/day i.p. for 4 consecutive days (19). In mice that received both treatments, the IL-12 injection regimen was started 1 day before the first HgCl₂ administration. Blood was obtained weekly by retro-orbital bleeding.

ANoA immunofluorescence

ANoA levels in serially diluted mouse serum were determined by indirect immunofluorescence as previously described (20). Sera diluted in PBS containing 1% BSA and 0.02% sodium azide were incubated with HEP-2 slides (Chemicon, Temecula, CA) for 30 min, and ANoA were detected with FITC-conjugated goat anti-mouse IgG1, IgG2a, or IgG2b Abs (Southern Biotechnology Associates, Birmingham, AL). The initial serum dilution was 1/100. The inverse of the highest serum dilution at which nucleolar fluorescence could be detected was defined as the ANoA titer.

B cell flow cytometry

On day 7 of treatment, spleens were removed from mice receiving HgCl₂ alone, IL-12 alone, or both HgCl₂ and IL-12 and were used to generate individual single cell suspensions. Cells were then treated with 0.165 M NH₄Cl to eliminate erythrocytes and washed three times in ice-cold staining medium (deficient RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) containing 10 mM HEPES, 3% BSA, and 0.1% NaN₃). Cells were resuspended in 0.1 ml of staining medium containing either 0.2 μg of FITC-R3A–6B2 anti-B220 mAb (PharMingen, San Diego, CA) or FITC-rat IgG2a isotype control mAb (PharMingen) and incubated for 15 min at 4°C. Cells were then washed three times in staining medium and analyzed by flow cytometry using a Coulter EPICS Elite apparatus (Coulter Electronics, Hialeah, FL).

ELISA for mouse serum IgG1, IgG2a, and IgG2b

Total serum IgG1, IgG2a, and IgG2b were determined using a sandwich ELISA adapted from a previously described method (16). For quantitation of IgG1, IgG2a, and IgG2b serum levels, plates were coated overnight at 4°C with goat anti-mouse IgE (Southern Biotechnology Associates, Birmingham, AL) diluted 2 μg/ml in carbonate buffer. Following three washes with PT buffer (PBS and 0.05% Tween), wells were blocked with PBTN (PBS containing 1% BSA, 0.05% Tween-20, and 0.02% sodium azide) for 30 min. Sera diluted 1/250,000 in PBTN were then added to wells and incubated at room temperature for 2 h. Samples were washed six times with PT, and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG1, IgG2a, or IgG2b secondary Ab (Southern Biotechnology Associates) diluted 1/4,000 in PBTN, was added to each well. Absorbance values were measured at 405 nm after 2 h. A standard curve was generated using varying concentrations (15–8000 pg/ml) of purified rIL-4 (National Cancer Institute Biologic Resources, Frederick, MD).

Renal Ig deposits

After 3 wk of treatment, kidneys were removed from mice that received HgCl₂, IL-12, or both. Bisected halves were snap-frozen in 1 to 2 ml of optimal cutting temperature (O.C.T.) compound (Miles, Inc., Elkhart, IN). Six-micron cryostat sections were prepared on a microtome (IEC, Needham Heights, MA) and fixed in acetone for 20 min. Sections were then soaked in PBS for 20 min and blocked with PBS containing 10% goat serum in a moist chamber. Following blocking, sections were incubated with dilutions of FITC-conjugated detection Abs specific for IgG1, IgG2a, or IgG2b. The primary dilution used was 1/50, and twofold dilutions were used until specific glomerular fluorescence was no longer detected. The inverse of the highest conjugate dilution at which fluorescence could be detected was defined as the glomerular Ig deposit titer (23).

Statistical analyses

These analyses were conducted with nonparametric tests using the GraphPad Prism software (version 2.01 (GraphPad Software, Inc., San Diego, CA)).

Results

IL-12 down-regulates Hg-induced ANoA production

Groups of A.SW mice (H-2b) were injected with HgCl₂ alone, IL-12 alone, or HgCl₂ and IL-12 as described in Materials and Methods. The production of highly specific autoantibodies against nucleolar Ags is the hallmark of Hg-induced autoimmunity, and we tested the sera from the various experimental groups for the presence of ANoA by immunofluorescence on HEP-2 cells. ANoA were not detectable at any time point in the group of mice that received IL-12 only. Although IgG class ANoA became detectable in the sera of both groups of mice receiving HgCl₂ injections (Fig. 1), the fluorescence staining intensity was, however, much lower in the group of animals that also received IL-12 (not shown). Since IL-12 can have different effects on various Ig isotypes, we determined the serum titers of the three major ANoA isotypes, i.e., IgG1, IgG2a, and IgG2b (Fig. 2). IgG1 ANoA were present after 1 wk in sera of mice receiving HgCl₂ at titers exceeding 10,000 and peaked at week 3. In contrast, sera from mice receiving both HgCl₂ and IL-12 showed no detectable IgG1 ANoA until week 3. ANoA levels in this group then slowly increased, but their mean titer was never >1,500 (Fig. 2). Likewise, both IgG2a and IgG2b

In vitro splenocyte stimulation

Spleens from day 7 treated mice were used to prepare single cell suspensions as described above. Cells were then washed several times in DMEM and resuspended in murine growth medium (DMEM containing 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES buffer, 5 × 10⁻³ M 2-ME, nonessential amino acids, and 10% FBS) at a concentration of 2 × 10⁶ cells/ml. Cells were then stimulated with 50 ng/ml PMA (Sigma) and 1 μM ionomycin (Sigma) for 19 h at 37°C. Cell supernatants were collected and assayed for IL-4 using the following ELISA.

IL-4 ELISA

IL-4 levels in supernatants were quantitated using a sandwich ELISA. Plates were coated overnight at 4°C with a rat anti-mouse IL-4 capture mAb (clone 11B11, PharMingen) diluted at 2 μg/ml in carbonate buffer. The capture Ab was washed out five times with PT, and the plate was blocked with PBTN for 30 min. Supernatants, either undiluted or diluted 1/5 in growth medium containing 0.02% sodium azide, were then added to wells and incubated at room temperature for 2 h. Absorbance values were measured at 405 nm after 16 h. A standard curve was generated using varying concentrations (15–8000 pg/ml) of purified rIL-4 (National Cancer Institute Biologic Resources, Frederick, MD).
ANoA titers steadily increased until week 4 and decreased thereafter in mice treated with HgCl₂ only, whereas the levels of these autoantibodies remained very low throughout in animals that received both HgCl₂ and IL-12 (Fig. 2).

**Effect of IL-12 injection on splenic B cells and serum IgG subclass levels**

After 1 wk, HgCl₂ treatment resulted in a significant increase (p < 0.05) in splenic B cell numbers compared with untreated animals (Fig. 3). Animals treated with both HgCl₂ and IL-12 or with IL-12 alone also displayed a moderate augmentation in splenic B cell numbers, although this increase was not significantly different from that in the control group (Fig. 3). To further characterize the effects of HgCl₂ and/or IL-12 on Ig production by B cells in A.SW mice, dilutions of sera were tested for IgG1, IgG2a, and IgG2b subclass Abs using a sandwich ELISA. While no overall changes in IgG1 were seen in sera of mice receiving four injections of IL-12, mice receiving HgCl₂ treatment showed an approximately 10-fold increase in IgG1 levels after 2 wk compared with preinjection levels (Fig. 4). Serum IgG1 levels in these mice peaked at week 3, declining rapidly thereafter. A.SW mice receiving both HgCl₂ and IL-12 had reduced levels of serum IgG1 after 2 wk compared with those in A.SW mice receiving HgCl₂ alone (Fig. 4).

Administration of HgCl₂ to H-2<sup>s</sup> mice also causes an increase in serum IgG2a levels. Indeed, after 3 wk of HgCl₂ injections, we observed an eightfold increase in serum IgG2a (Fig. 4) compared with preinjection levels, confirming previous findings (16). Injection with IL-12 alone also resulted in an increase in serum IgG2a after 3 wk. Mice receiving both HgCl₂ and IL-12 had higher levels of serum IgG2a than either of the other two groups by week 3 (Fig. 4). Serum levels of IgG2b remained relatively unchanged for all three groups of mice throughout the course of the experiment (Fig. 4).

It is worth emphasizing that in mice receiving HgCl₂ only, ANoA production did not closely follow the time course of IgG subclass production in the serum of A.SW mice. IgG1 and IgG2a ANoA developed slightly later than the total serum increases in the corresponding isotypes, and IgG2b ANoA were readily detectable even though there was no significant increase in total serum IgG2b.

**FIGURE 1.** ANoA in serum of HgCl₂-injected A.SW mice. Representative HEp-2 staining pattern obtained with serum diluted 1/100 and detected with an FITC-conjugated goat anti-mouse IgG1 conjugate.

**FIGURE 2.** IL-12 decreases ANoA induction in HgCl₂-injected A.SW mice. ANoA were detected by immunofluorescence on HEp-2 cells using isotype-specific FITC conjugates. Results are expressed as serum titers, i.e., inverse of the highest serum dilution that yielded nucleolar fluorescence. A.SW mice that received only IL-12 (no HgCl₂) did not develop ANoA. Ten mice were included in each experimental group. Comparisons between the two treatment groups were performed using the Mann-Whitney test. Statistically significant differences are indicated by an asterisk.

**FIGURE 3.** Splenic B cell numbers in A.SW mice that received HgCl₂ and/or IL-12. B220<sup>+</sup> cells were identified and quantified as described in Materials and Methods. Measurements were conducted after 7 days of HgCl₂ and/or IL-12 treatment. Three mice were included in each experimental group. Comparisons among the experimental groups were performed using the Kruskal-Wallis and Dunnett tests. B cell numbers were significantly increased (p < 0.05) in the group treated with HgCl₂.
These and previous observations support the view that ANoA are not merely the product of nonspecific polyclonal activation, but result from a specific Ag-driven response (20).

IL-12 does not inhibit the Hg-induced increase in serum IgE

To determine the effect of IL-12 on the Hg-induced increase in serum IgE, A.SW mice were treated as described above, and total serum IgE was measured by ELISA at various time points after the beginning of the injections. IL-12 alone did not affect the overall levels of serum IgE (Fig. 5). In contrast, by week 2, mice receiving HgCl₂ displayed a 38-fold increase in serum IgE over week 0 levels. Mice receiving HgCl₂ and IL-12 also showed increased levels of IgE that were not significantly different (p > 0.05) from those in mice that received HgCl₂ only. The serum IgE peak, however, was delayed in the HgCl₂ plus IL-12 group (week 3) compared with that in the HgCl₂ only group (week 2).

IL-12 potentiates HgCl₂-induced IL-4 production

Several studies have shown that in susceptible animal strains, HgCl₂ can elicit IL-4 production both in vivo and in vitro (13, 14, 18). We therefore investigated whether IL-12 treatment affected the HgCl₂ induction of IL-4 secretion in A.SW mice. After 1 wk of treatment with HgCl₂ and/or IL-12, A.SW splenocytes were restimulated in vitro with PMA plus ionomycin, and IL-4 production in the supernatant was measured by ELISA. The results presented in Figure 6 show that in vivo treatment with HgCl₂ induced IL-4 production by A.SW splenocytes and that this induction was increased in animals receiving both HgCl₂ and IL-12 (p < 0.05). In contrast, splenocytes from animals receiving IL-12 only without HgCl₂ produced IL-4 levels comparable to those in untreated controls.

IL-12 does not significantly affect Hg-induced renal Ig deposits

Renal Ig deposits were detected by immunofluorescence performed on kidney cryostat sections prepared from A.SW mice injected with HgCl₂ alone, IL-12 alone, or HgCl₂ and IL-12. IgG1, IgG2a, and IgG2b deposits were detected 3 wk after the start of the injections using isotype-specific fluorescent conjugates. Since even normal mice showed a significant level of Ig background staining, kidney cryostat sections from untreated A.SW mice were also used as controls. Compared with normal mice, these background levels of renal deposits were not significantly increased in mice treated with IL-12 only (and were most pronounced for the IgG2a subclass; Fig. 7). IgG1 renal deposits were significantly higher in both groups of mice that received HgCl₂ compared with those in mice that received IL-12 alone (Fig. 5). However, there was no significant difference in the levels of IgG1 deposits between the groups receiving HgCl₂ alone or HgCl₂ plus IL-12. The IgG2a and IgG2b deposits were not significantly different among the three experimental groups (Fig. 7). These results indicate that IL-12 treatment did not alter Ig deposition in mice injected with HgCl₂.
cytes and mast cells (13, 14), and IL-4 is produced in vivo after Hg injections (15). Recent results suggest that Hg-treated Brown-Norway rats are deficient in the expression of IL-12, the main Th1 regulatory cytokine, compared with the resistant Lewis strain (30). We therefore investigated whether the administration of IL-12, a cytokine critical in favoring the establishment of a Th1 over a Th2 response, would affect the development of Hg-induced autoimmunity. Our results indicate that administration of rIL-12 at the onset of the syndrome inhibits the Hg-induced increase in serum IgG1 and potentiates the IgG2a induction, but does not significantly affect the IgE increase. This result was partly unexpected, since the induction of IFN-γ by IL-12 can down-regulate class-switching to both IgG1 and IgE (31). Nevertheless, under certain circumstances, IL-12 can enhance IgE synthesis. In CBA/J mice, administration of high doses of IL-12 suppresses IgE responses after phospholipase A2 immunization, whereas low doses of IL-12 lead to elevated levels of IgE Abs against the same immunogen (31). When keyhole limpet hemocyanin is used as an immunogen in the same CBA/J strain, both high and low doses of IL-12 suppress IgE responses. In contrast, in BALB/c mice, both high and low doses of IL-12 enhance the synthesis of IgE directed against keyhole limpet hemocyanin (31). Thus, the ability of IL-12 to influence Th2 cytokine-dependent IgE production varies according to the immunologic setting. These apparently paradoxical results can be partly explained by recent developments in our understanding of Th cell differentiation. IL-12 and IL-4 control the development of Th1 and Th2 cell types, respectively. Nevertheless, in the presence of both cytokines, IL-4 effects are dominant, and IL-12 even enhances the development of Th2 cytokine-producing cells (32).

Likewise, we observed an increase in Hg-induced IL-4 production in mice treated with IL-12. In susceptible strains, HgCl2 strongly induces IL-4 synthesis by T cells and mast cells, both in vivo and in vitro (13–15). Thus, treatment of mice with both HgCl2 and IL-12 may favor the development of Th2 cells with a mixed Th0 phenotype. The balance of Th1 and Th2 cytokines induced by these treatments may determine the differential effects of IL-12 on IgG1 and IgE levels, since switching to these two isotypes is differentially controlled (33).

Our results are in contrast with those of Ochel et al., who found that anti-IL-4 treatment did not prevent the development of ANoA, but shifted the response from the IgG1 to the IgG2a and IgG2b isotypes (16). In contrast, anti-IL-4 treatment prevented both IgG1 and IgE hyperglobulinemia (16). A recent study from the same group showed that IFN-γ treatment can partly prevent the HgCl2-induced increase in serum IgE, but does not affect the formation of ANoA (34). Therefore, three “Th1-promoting” treatments (anti-IL-4, IFN-γ, and IL-12) differed in their effects on the various manifestations of Hg-induced autoimmunity. In view of the current interest in manipulating immune responses by affecting the Th1-Th2 balance, these combined observations suggest that apparently analogous biologic agents can have largely different results on Th-dependent manifestations of autoimmune disease.

In susceptible strains, Ig deposits form in the kidneys of Hg-treated animals, resulting in a transient proteinuria. The exact mechanisms of kidney damage are not understood, and there is even controversy as to whether these animals develop true glomerulonephritis (9). In our study, IL-12 treatment did not significantly affect the kidney deposit levels of the various IgG isotypes. These results indicate that ANoA do not contribute significantly to the nephritis, since their titers were dramatically decreased in IL-12-treated mice. Our findings are in agreement with those of several studies that concluded that ANoA are not required for the presence of Ig deposits in Hg-induced autoimmunity. For instance, backcross studies between susceptible SJL and resistant C57Bl/6
mice have shown that mercury-treated mice can develop renal IgG deposits without concomitant serum ANaA (35). Conversely, the presence of ANaA does not necessarily result in the development of immune complex deposits. Susceptible mice treated orally with HgCl₂ (36) or injected with silver nitrate produce ANaA without renal IgG deposits (37). Therefore, ANaA production does not correlate with renal immune complex deposition (38).

Murine chronic graft-vs-host disease (GVHD) shares several features with heavy metal-induced autoimmunity, suggesting that common mechanisms underlie both syndromes. Thus, the injection of parental spleenocytes into (C57Bl/6 × DBA/2) F₁ mice results in hyperglobulinemia, increased secretion of Th2 cytokines, and autoantibody production. Pretreatment of these mice with IL-12 abolishes most of the manifestations of chronic GVHD, but actually results in a more lethal and acute form of GVHD (39, 40). Furthermore, the effect of IL-12 on the manifestations of chronic GVHD differs somewhat from its effect on Hg-induced autoimmunity. For instance, IL-12 decreases autoantibody production in both models, but whereas it prevents the induction of all serum Ig isotypes in chronic GVHD (40), it prevents only the IgG₁ increase in the Hg-induced syndrome. These results indicate that the cytokine interactions that control metal-induced autoimmunity and chronic GVHD are only partly similar. Furthermore, our observations support the view that the various manifestations of Hg-induced autoimmunity are independently regulated.

Acknowledgments

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