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Inhibitory Signal Override Increases Susceptibility to Mercury-Induced Autoimmunity

Yan Zheng and Marc Monestier

After exposure to subtoxic doses of heavy metals such as mercury, H-2b mice develop an autoimmune syndrome consisting of the rapid production of IgG autoantibodies that are highly specific for nucleolar autoantigens and a polyclonal increase in serum IgG1 and IgE. In this study, we explore the role of two inhibitory immunoreceptors, CTLA-4 and FcγRIIB, in the regulation of mercury-induced autoimmunity. In susceptible mice treated with mercuric chloride (HgCl2), administration of a blocking anti-CTLA-4 Ab resulted in a further increase in anti-nucleolar autoantibodies and in total serum IgG1 levels. Furthermore, in some DBA/2 mice, which are normally resistant to heavy metal-induced autoimmunity, anti-CTLA-4 treatment leads to the production of anti-nucleolar Abs, thereby overcoming the genetic restriction of the disease. In mice deficient for the FcγRIIB, HgCl2 administration did not trigger autoantibody production, but resulted in an increase in IgE serum levels. Taken together, these results indicate that different inhibitory mechanisms regulate various manifestations of this autoimmune syndrome. The Journal of Immunology, 2003, 171: 1596–1601.

Mice or rat strains expressing certain MHC Ags are susceptible to the heavy metal induction of a complex autoimmune syndrome (1–3). In susceptible H-2b mice, subtoxic doses of HgCl2 induce an autoimmune dysfunction characterized by the production of anti-nucleolar Abs (ANoA), lymphoproliferation, and hyperglobulinemia (especially pronounced for IgG1 and IgE). The increases in serum Ig peak 2–3 wk after the beginning of the HgCl2 injections, whereas ANoA can persist for several months after the induction phase.

In addition to Ag-specific signals through the TCR, T cells require additional Ag-nonspecific costimulatory signals for activation. The B7 molecules, B7-1 (CD80) and B7-2 (CD86), provide costimulatory signals for the development of immune responses to foreign and self-Ags. The CD28 and CTLA-4 (CD152) molecules expressed on T cells are the specific receptors for the B7 molecules on APCs. CD28/B7 interactions are important for augmenting and sustaining T cell responses, and can have multiple effects on the production of various cytokines, chemokines, and antiapoptotic proteins (4, 5). In contrast, the CTLA-4/B7 interaction dampens T cell activation (6). Blockade of CTLA-4 increases T cell responses in vitro (7, 8) and in vivo (9), augments antimurine immunity (10), and exacerbates autoimmune diseases (11, 12). Our recent studies have demonstrated that B7/CD28-mediated costimulation is critical for the development of mercury-induced autoimmunity (13). One of aims of the present work is to investigate the role of the inhibitory receptor CTLA-4 in the development of mercury-induced disease and to determine whether the blockade of signaling through this molecule selectively affects the manifestations of this syndrome.

In addition to CD28/CTLA-4, FcRs offer another paradigm for balancing activation and inhibitory signaling in the expanding family of activating/inhibitory receptor pairs found in the immune system (14). The FcRs are a family of hemopoietic cell surface molecules, which include receptors that can either stimulate or inhibit cellular responses upon the binding of Ab-Ag complexes. Various functions can be triggered by the activating receptors. FcγRIII or FcεRI (15–17). These activation responses are balanced by the widely expressed inhibitory receptor FcγRIIB. FcγRIIB suppresses B cell, mast cell, and macrophage activation (18). A recent study showed that FcγRIIB-deficient (FcγRIIB−/−) mice on a resistant background (H-2b) became susceptible to collagen-induced arthritis upon immunization with collagen type II (19). Some other studies also indicate that FcγRIIB physiologically acts as a negative regulator of immune complex-activated activation and may function in vivo to suppress autoimmunity by regulating both B cell responses and effector cell activation (17, 20–22). Therefore, another aim of this study is to elucidate whether FcγRIIB can affect the development of HgCl2-induced autoimmune disease.

Materials and Methods

Mice

Female A.SW/SnJ (H-2b), DBA/2 (H-2b), FcγRIIB−/− (B6; 129S-Fcgr2b−/−), and B6129SF2J (H-2b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal facilities. All mice used in our experiments were at least 2 mo old.

Antibodies

The blocking hamster anti-CTLA-4 Ab-producing hybridoma (9H10) was a kind gift from Dr. J. Allison (University of California, Berkeley, CA) (7). mAbs were purified from culture supernatants by affinity chromatography over a protein G column. Hamster IgG from Rockland (Gilbertsville, PA) was used as a control for the anti-CTLA-4 treatment experiments.

HgCl2 and Ab treatment

Mercury-induced autoimmunity was induced in groups of mice according to a standard protocol by s.c. injection (30 μg of HgCl2 in 100 μl of sterile PBS) three times weekly (23). In addition to HgCl2, some groups of mice...
To assess the roles of CTLA-4/B7 interaction in HgCl₂-induced autoimmunity during the early induction phase

**ANOa immunofluorescence**

Serum ANOa levels were determined by indirect immunofluorescence, as described previously (23). Sera diluted in PBN (PBS containing 1% BSA and 0.02% sodium azide) were incubated with Hep-2 slides (Antibodies Incorporated, Davis, CA) for 30 min, and ANOa were detected with FITC-conjugated goat anti-mouse IgG1 Abs (Southern Biotechnology Associates, Birmingham, AL). The inverse of the highest serum dilution at which nucleolar fluorescence could be detected was defined as the ANOa titer.

**ELISA for mouse serum IgG1**

Total serum IgG1 levels were determined using a sandwich ELISA adapted from a previously described method (23). Briefly, plates were coated overnight at 4°C with goat anti-mouse Ig κ (Southern Biotechnology Associates) diluted to 2 µg/ml in carbonate buffer. Following three washes with PT buffer (PBS plus 0.05% Tween 20), plates were blocked with PBTN (PBS containing 1% BSA, 0.05% Tween 20, and 0.02% sodium azide) for 30 min. Sera diluted 1/50,000 in PBTN were then added to wells and incubated at room temperature for 2 h. Samples were washed out six times with PT, and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG1 secondary Ab (Southern Biotechnology Associates) diluted 1/4000 in PBTN was added for 1.5 h. Secondary Ab was washed out with two washes of each of PT and AP substrate buffer (10 mM diethanolamine and 0.5 mM MgCl₂ in pH 9.2). p-Nitrophenylphosphate substrate (1 mg/ml in AP buffer) was then added and allowed to develop for 20 min. Absorbance values were read at 405 nm. A standard curve was generated using varying concentrations (1.6–200 ng/ml) of ASWU1 (IgG1) mAb (24).

**ELISA for mouse serum IgE**

Total serum IgE levels were determined using a sandwich ELISA that has been described previously (23). Briefly, plates were coated overnight at 4°C with a rat anti-mouse IgE capture mAb (clone R35-72; BD Pharmingen, San Diego, CA) diluted to 2 µg/ml in carbonate buffer. Following several washes and a blocking step with PT, sera diluted 1/100 in PBTN were then added to wells and incubated at room temperature for 2 h. After several washes, the secondary Ab, biotinylated rat anti-mouse IgE (clone R35-92; BD Pharmingen) diluted to 2 µg/ml in PBTN was then added and allowed to develop for 20 min. Absorbance values were read at 405 nm. A standard curve was generated using varying concentrations (3–400 ng/ml) of purified mouse IgE (clone IgE-3; BD Pharmingen).

**Statistical analyses**

These analyses were conducted with nonparametric tests using the GraphPad Prism software (version 3.0; GraphPad Software, San Diego, CA). Comparisons among three experimental groups were performed using the Kruskal-Wallis and Dunnnett tests. Comparisons between two experimental groups were performed using the Mann-Whitney test.

**Results**

**Blockade of CTLA-4 pathway exacerbates HgCl₂-induced autoimmunity during the later disease development**

To further examine the role of CTLA-4, we postponed the Ab treatment under protocol 1, mice receiving HgCl₂ and anti-CTLA-4 Abs developed significantly (p < 0.05) higher serum total IgG1 levels than mice receiving either HgCl₂ alone or HgCl₂ plus isotype-matched control Abs, although the serum total IgE levels among these three groups were not significantly different (Fig. 2). There were no differences among these three groups at other time points (data not shown).

**Blockade of CTLA-4 pathway exacerbates HgCl₂-induced autoimmunity during the later disease development**

To further examine the role of CTLA-4, we postponed the Ab administration to the later phase of disease development. In protocol 2 (Fig. 1), groups of A.SW mice received HgCl₂ injections three times per week throughout the entire experiment. Anti-CTLA-4 mAbs or control hamster IgG were administered on days 21, 24, and 27, respectively. On day 28, as was the case with the previous Ab treatment under protocol 1, mice receiving HgCl₂ and anti-CTLA-4 mAbs developed significantly (p < 0.05) higher antinucleolar autoantibody (Fig. 3) than mice treated with HgCl₂ or HgCl₂ plus isotype-matched control Abs. Serum total IgG1 levels of mice receiving anti-CTLA-4 treatment were not significantly different, compared with the other two groups (Fig. 3). No differences were observed among these three groups at other time points (data not shown).

**A.** Anti-CTLA-4 mAb or hamster IgG

**B.** HgCl₂ s.c. (3x weekly)

Protocol 1:

| Day | 0 | 7 | 10 | 13 | 14 |

Protocol 2:

| Day | 0 | 7 | 10 | 13 | 14 | 21 | 24 | 27 | 28 |

**FIGURE 1.** Time course of HgCl₂ and anti-CTLA-4 Ab injection protocols. In both protocols 1 and 2, mice received s.c. injections of 30 µg of HgCl₂ in 100 µl of sterile PBS three times weekly throughout the duration of the experiment. In protocol 1, three injections of anti-CTLA-4 mAb or control hamster IgG Ab were administered at days 7, 10, and 13. In protocol 2, Abs were administered at days 21, 24, and 27 (6, 10). In all cases, Abs were administered i.p., and mice received 100 µg of each Ab in 500 µl of sterile PBS per injection.

Because DBA/2 (H-2b) is a strain believed to be completely resistant to mercury-induced autoimmunity (25, 26) and given that blocking CTLA-4 seems to exacerbate mercury-induced autoimmunity in susceptible strains (A.SW), we hypothesized that, in resistant DBA/2 mice, CTLA-4 may also regulate the susceptibility to the disease. Therefore, we conducted another series of experiments with groups of DBA/2 mice according to protocol 1 (Fig. 1). Briefly, mice received HgCl₂ injections three times per
week throughout the entire experiment. Anti-CTLA-4 mAbs or control hamster IgG were administered on days 7, 10, and 13, respectively. On day 14, we conducted an indirect immunofluorescence assay to check the autoantibody production in the three groups of mice (Figs. 4 and 5). Surprisingly, three of five mice that received HgCl₂ and anti-CTLA-4 mAb treatment developed ANoA with typical antinucleolar staining pattern, similar to the positive control using antinucleolar ASWU1 (IgG1) mAb (24). However, none of the mice receiving HgCl₂ alone or both HgCl₂ and control hamster IgG Abs developed ANoA at all, which is consistent with previous findings (25). To assess the specificity of the autoantibodies in DBA/2 mice that received both anti-CTLA-4 and HgCl₂, we also performed immunoblotting experiments against nucleolar extracts (data not shown). None of the sera from these mice reacted with fibrillarin or with other Ags on the blot. We believe that this lack of reactivity is probably due to the relatively low sensitivity of this method and to the low levels of autoantibodies in DBA/2 mice. Indeed, the ANoA titers barely exceeded 50 in DBA/2 mice treated with HgCl₂ (Figs. 2 and 3). At the same time, mice treated with HgCl₂ and anti-CTLA-4 mAbs had slightly higher serum IgG1 levels, but significantly (p < 0.05) higher serum IgE levels than mice receiving HgCl₂ or HgCl₂ plus isotype-matched control Abs (Fig. 5), although the increase in the levels of serum total IgG1 and IgE in DBA/2 mice are much lower than seen in susceptible A.SW mice and may not be biologically meaningful.
At other time points, no significant differences were observed among three groups (data not shown).

HgCl2-induced autoimmunity in FcγRIIB−/− mice

We also conducted a series of experiments with FcγRIIB−/− mice to determine whether this inhibitory pathway may regulate HgCl2-induced autoimmunity. Groups of FcγRIIB−/− or control B6129SF2/J mice received HgCl2 injections three times per week throughout the entire experiment (23). The increase in serum IgG1 was mild in both groups, as expected, due to their resistant H-2b background. However, no significant differences were observed with serum IgG1 levels between these two groups (Fig. 6). At the same time, although B6129SF2/J mice also developed a mild increase in the levels of serum IgE, FcγRIIB−/− mice developed significantly higher serum IgE levels (Fig. 6). Moreover, when tested by immunofluorescence, neither group of mice developed ANoA (not shown).

Discussion

To maintain an appropriate immune response, the activation of the immune system must be balanced with an inhibitory pathway. A typical example is the CD28/CTLA-4:B7-1/B7-2 costimulation pathway. Although CD28 augments T cell activation, differentiation, and survival, CTLA-4-deficient mice die within 1 mo of birth with severe T cell proliferative disorders (27). Autoimmune diseases may be associated with a deficiency of inhibitory signaling, suggesting an essential role for activating/inhibitory systems during immune regulation (18, 28). The importance of CD28/CTLA-4:B7-1/B7-2 costimulation is attested by experiments showing that complete blockade of this pathway either by a CTLA-4-Ig fusion protein (29) or by a combination of anti-B7-1/B7-2 Abs (13) results in the complete abolition of all manifestations of mercury-induced autoimmunity.

Although CD28 is constitutively expressed on T cells, CTLA-4 is only expressed on activated T cells and binds to the same ligands as CD28, but with a much higher affinity. This suggests that CTLA-4 may preferentially act as a terminator of immune responses after T cells have been activated. In this study, we demonstrate that blockade of CTLA-4 negative signaling by anti-CTLA-4 mAb exacerbates HgCl2-induced autoimmunity both in the early and late phases of the syndrome. Although anti-CTLA-4 mAb treatment had no effect on serum total IgE levels, it temporarily but significantly increased the serum total IgG1 and ANoA levels immediately after the last dose of Ab treatment. The transitory effect of the treatment is probably due to the limited effect of the Ab itself or to the anti-hamster Abs produced by the mice that received the treatment. Overall, our results support the view that CTLA-4 is a negative regulator of autoimmune diseases (11) and are consistent with previous findings that correlated the detection...
of anti-CTLA-4 Abs in patients’ sera with their systemic autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis (30).

Furthermore, the similar levels of serum total IgE between anti-CTLA-4-treated and control groups also support our previous hypothesis that different regulatory mechanisms control the various aspects of the syndrome (31). Most of the increase in serum IgG1 and IgE Igs during HgCl2-induced autoimmunity is polyclonal in nature and not targeted against a specific Ag. Our data suggest that CTLA-4 blockade may breakag the Ag-specific T cell tolerance and result in autoimmune production, although the exact molecular mechanisms governing the CTLA-4 activity are still unclear. Cytokines such as IFN-γ or IL-4 are probably critical in these mechanisms, but unfortunately, the serum cytokines levels in our experiments were below the detection levels of commercial assays (data not shown). Recently, Hurwitz et al. (32) have shown that CTLA-4 blockade resulted in a preferential increase in the frequency of Ag-specific T cells secreting the Th-1 cytokine IFN-γ. Moreover, Kono et al. (33) have suggested that IFN-γ is essential for the production of ANoA during HgCl2-induced autoimmunity. These findings are in agreement with our data showing that blockade of CTLA-4 exacerbates specific manifestations of HgCl2-induced autoimmunity, such as autoantibody production and increase in serum IgG1 levels, but does not affect serum IgE levels. HgCl2-induced autoimmunity is characterized by hyperimmunoglobulinemia, primarily of IgG1 and IgE isotypes (34). However, our data show disassociation between serum IgG1 and IgE levels upon CTLA-4 blockade in this model. One study has suggested that, at higher concentration, IFN-γ has minor inhibitory effects on isotype switching to IgG1 and greater effects on IgE (35). In fact, as we mentioned before, it has been shown that CTLA-4 blockade increases IFN-γ production by Ag-specific T cells (32). Our data are consistent with the view that the disassociation between serum IgG1 and IgE levels we observed is primarily due to the production of IFN-γ upon CTLA-4 blockade.

Previous studies have shown that blockade of CTLA-4 exacerbates experimental autoimmune encephalomyelitis in susceptible mice (11, 36) and renders resistant mice susceptible to experimental autoimmune encephalomyelitis (32). In our model, mercury-treated mice from strains carrying the H-2s haplotype develop ANoA, whereas strains with the H-2β and H-2d haplotypes are resistant to induction of ANoA (37). Consistently, we have shown that CTLA-4 blockade exacerbates HgCl2-induced autoimmunity in susceptible H-2β mice. Moreover, in our experiment with resistant DBA/2 mice (H-2β), we indeed observed that HgCl2 alone or HgCl2 plus isotype-matched control Abs did not induce ANoA production. Importantly, we found that CTLA-4 blockade by anti-CTLA-4 mAb rendered some DBA/2 mice susceptible to HgCl2-induced autoimmunity, overcoming the H-2 haplotype restriction. DBA/2 is probably the most resistant strain to mercury-induced autoimmunity, because it does not produce autoantibodies and displays a very limited increase in serum Igs after HgCl2 administration (26). Some strains, such as BALB/c (which is H-2d like DBA/2), possess an intermediary phenotype, i.e., they do not produce ANoA following HgCl2 administration, but they display a significant increase in serum Igs with renal deposits. In future studies, it will be interesting to assess how anti-CTLA-4 treatment affects mercury-induced autoimmunity in BALB/c mice and whether CTLA-4 plays a role in the susceptibility differences between DBA/2 and BALB/c.

The results presented in this study directly implicate CTLA-4 as one of critical factors controlling the susceptibility to HgCl2-induced autoimmunity. Other studies have suggested two possible mechanisms to our findings. One possibility is that CTLA-4 may act as an attenuator of the T cell activation threshold (38, 39). Blockade of CTLA-4 lowered this threshold and increased the number of activated T cells. Alternatively, CTLA-4 may be critical for the clonal expansion of Ag-specific T cells (40).

FcγRIIB is another member of the immune inhibitory receptor family with a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (41). On the C57BL/6 background, mice deficient in FcγRIIB spontaneously develop anti-nuclear Abs followed by a fatal glomerulonephritis (28). A recent study indicates that the loss of tolerance in FcγRIIB−/− mice is functionally linked to the Sle1 interval (42). Furthermore, the disease in FcγRIIB−/− mice is modulated by other autoimmunity genes. The yaa gene enhances the syndrome by changing the specificity of the autoantibodies, whereas the lpr mutation decreases renal disease in these animals without affecting autoantibody levels (42). Our studies with HgCl2-treated FcγRIIB−/− mice only indicated an increase in serum IgE levels and no detectable ANoA production. These data suggest that CTLA-4 and FcγRIIB have distinct regulatory functions during HgCl2-induced autoimmunity. We propose that, in our HgCl2-induced autoimmunity model, ANoA production might be CTLA-4 dependent, but FcγRIIB independent. At the same time, serum IgE levels might depend on FcγRIIB instead of the CTLA-4 pathway. This would explain why in our experiments, blockade of CTLA-4 in normal mice did not affect the increase in serum IgE levels.

In summary, our data indicate that CTLA-4 has a crucial negative regulatory function in HgCl2-induced autoimmunity. Manipulation of this regulatory pathway might have some therapeutic
benefits for the immunotherapy of human autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis.

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References