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*J Immunol* 2005; 175:2018-2025; doi: 10.4049/jimmunol.175.3.2018
http://www.jimmunol.org/content/175/3/2018

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IL-1 Receptor Antagonist-Mediated Therapeutic Effect in Murine Myasthenia Gravis Is Associated with Suppressed Serum Proinflammatory Cytokines, C3, and Anti-Acetylcholine Receptor IgG1

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In myasthenia gravis (MG), TNF and IL-1β polymorphisms and high serum levels of these proinflammatory cytokines have been observed. Likewise, TNF and IL-1β are critical for the activation of acetylcholine receptor (AChR)-specific T and B cells and for the development of experimental autoimmune myasthenia gravis (EAMG) induced by AChR immunization. We tested the therapeutic effect of human recombinant IL-1 receptor antagonist (IL-1ra) in C57BL/6 mice with EAMG. Multiple daily injections of 0.01 mg of IL-1ra administered for 2 wk following two AChR immunizations decreased the incidence and severity of clinical EAMG. Furthermore, IL-1ra treatment of mice with ongoing clinical EAMG reduced the clinical symptoms of disease. The IL-1ra-mediated suppression of clinical disease was associated with suppressed serum IFN-γ, TNF-α, IL-1β, IL-2, IL-6, C3, and anti-AChR IgG1 without influencing total serum IgG. Therefore, IL-1ra could be used as a nonsteroidal drug for the treatment of MG. The Journal of Immunology, 2005, 175: 2018–2025.

M yasthenia gravis (MG)4 and its animal model experimental autoimmune MG (EAMG) are characterized by an autoimmune response against the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction (NMJ) (1–4). AChR-specific T cells induce AChR-specific B cells via cognate interaction with B cells and cells of the immune response to produce anti-AChR Abs. Binding of high affinity anti-AChR Ab at the NMJ activates the classical complement cascade and destroys AChR in the NMJ, thus resulting in muscle weakness (1–7).

IFN-γ, TNF-α, IL-6, IL-12, IL-18, IL-1, IL-5, and IL-10 have been shown to play a disease-promoting role in EAMG (8–21). In MG, TNF and IL-1β polymorphisms and high serum levels of these proinflammatory cytokines have been observed (22, 23). We are evaluating the therapeutic effect of nonsteroidal drugs in EAMG and MG. Soluble recombinant human TNFR:Fc administration reduced the severity of clinical signs in C57BL/6 mice with established clinical EAMG (24) and in steroid-dependent MG patients (39).

The IL-1 family is an important part of the innate immune system, which regulates functions of the adaptive immune system. Early in the immune response, it increases the expression of a wide variety of other cytokine genes including IL-6 and IL-12, and down-regulates the constitutive expression of several housekeeping genes and receptor genes including its own and receptors of TNF (25, 26). The IL-1 family consists of two agonists (IL-1α and IL-1β), two receptors (biologically active IL-1R type I and inert IL-1R type II), and a specific receptor antagonist (IL-1 receptor antagonist (IL-1ra)). IL-1α is a specific, high affinity antagonist that inhibits IL-1α and IL-1β and thus controls the activity exerted by IL-1.

The balance between IL-1 and IL-1ra in local tissues plays an important role in the susceptibility to and severity of many diseases (25, 26). The beneficial effects of administration of recombinant IL-1ra in many experimental animal models of inflammation, host defense, and the neuroimmunooendocrine system diseases have been reported (27–34). The therapeutic effect of IL-1ra in a classical Ab and complement-mediated autoimmune disease has not yet been investigated. In this study, we demonstrate the therapeutic efficacy of IL-1ra in EAMG and the possible mediation of this therapeutic effect.

Materials and Methods

AChR and mice

AChR was purified from the electric organ of Torpedo californica (T-AChR) by a neurotoxin affinity column (35, 36). T-AChR α-chain peptide (α146–162) was synthesized at the M. D. Anderson Cancer Center (Houston, TX). Seven- to 8-wk-old male C57BL/6 mice were purchased from The Jackson Laboratory. Mice were maintained in the viral Ab-free barrier facility at the University of Texas Medical Branch (Galveston, TX) and maintained according to the Institutional Animal Care and Use Committee Guidelines.
Immunization and evaluation of EAMG

For disease prevention studies (experiment 1), 18 C57BL/6 mice were anesthetized and immunized s.c. (foot pad and shoulder) with T-AChR (20 μg) emulsified in CFA (Difco). They were boosted on day 30 with 20 μg of T-AChR. Five mice were i.p. injected five times (due to the very short half-life of IL-1ra) per day for 2 wk with 0.01 mg of IL-1ra, and four mice were left as controls. Figure 1 shows the kinetics of the incidence and mean severity of clinical EAMG in IL-1ra or placebo treated mice in prevention experiments (experiment 1) after second AChR immunization (*, p < 0.05 using Fisher’s exact probability test for all comparisons from day 3 after second AChR immunization to the end of the experiment). The mean clinical scores (severity) of mice injected with 0.01 mg of IL-1ra were significantly lower than those of placebo injected mice (from day 3 to the end of the experiment) after second AChR immunization (*, p < 0.05, Student’s t test).

**FIGURE 1.** The kinetics of the incidence and mean severity of clinical EAMG. Kinetics of the accumulated clinical EAMG incidence (A and C) and mean clinical severity (B and D) in IL-1ra or placebo treated mice in prevention experiments (experiment 1 (A and B) and experiment 2 (C and D)) (*, p < 0.05 using Fisher’s exact probability test for all comparisons from day 3 after second AChR immunization to the end of the experiment). The mean clinical scores (severity) of mice injected with 0.01 mg of IL-1ra were significantly lower than those of placebo injected mice (from day 3 to the end of the experiment) after second AChR immunization (*, p < 0.05, Student’s t test).

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**FIGURE 2.** Serum anti-AChR IgG, IgG1, IgG2b, and IgG2c levels and total serum IgG, IgG1, and IgG2b isotype levels of IL-1ra or placebo treated mice (prevention experiment 1) are shown. Serum samples obtained from individual IL-1ra- and placebo-injected mice on day 60 after the first AChR immunization were assessed for anti-mouse muscle AChR IgG by radioimmunoassay. Serum anti-mouse AChR isotype and serum total IgG, IgG1, and IgG2b levels were measured by ELISA (*, p < 0.05 using Student’s t test for the comparison of 0.001 mg group vs each of the other two groups). Bars indicate medians.
were given 0.001 mg of IL-1ra, starting on day 0. As placebo control, nine mice were injected with 0.01 mg/ml polyvinyl pyrrolidone (stabilizer for IL-1ra lyophilization). In experiment 2, the previous experiment was repeated (except only a 0.01-mg dose of IL-1ra was tested) in 20 C57BL/6 mice. Mice were screened for clinical EAMG using the following modified score: grade 0, mouse with normal muscle strength and activity when placed on table top; grade 0.5, normal at rest, weak paw grip, or backward movement after 20–30 paw grips (paw exercise) on cage top grids; grade 1, normal at rest, with muscle weakness characteristically shown by hunched-back posture, and weak grip or backward movement after

FIGURE 3. Reduced serum C3 levels in IL-1ra-injected mice (prevention experiment 1) are shown. Sera derived from individual IL-1ra- and placebo-injected mice on days 15 and 60 after the first AChR immunization were assayed for C3, C1q, C1q-CIC, and C3-C1C by ELISA (*, p < 0.05 by Student's t test for the comparison of 0.01 mg group vs each of other two groups). Bars indicate medians.

FIGURE 4. Suppressed serum IL-1β, IL-2, IL-6, IFN-γ, and TNF-α production in IL-1ra-injected mice (prevention experiment 1). *, p < 0.05, ****, p < 0.0001 by Student's t test for the comparisons of 0.01 mg and/or 0.001 mg group vs placebo group. Bars indicate medians.
exercise; grade 1.5, similar to grade 1 with tremulous movements and chin on the floor and temporary difficulty in raising head; grade 2, showing grade 1.5 symptoms and signs after a minute of walking/slow running on the table top without paw exercise; grade 2.5, grade 2 weakness obvious soon after placing the mouse on the table top; grade 3, dehydrated and moribund with or without closure or secretions of the eyes with grade 2.5 weakness; grade 3.5, imminent death (death within a few hours); grade 4, dead. All mice were bled periodically to collect serum for evaluation of anti-AChR Ab levels. Thirty days after the boost with T-AChR, mice were euthanized; the draining lymph node cells (LNC) and carcasses from individual mice were collected (36).

For treatment study, 24 of 60 adult C57BL/6 male mice developing a grade 1.5 or higher clinical EAMG after two or three immunizations with T-AChR in CFA were divided into two groups of 12 mice each with equivalent clinical grades. Mice were injected daily with 0.01 mg of IL-1ra or placebo for 2 wk. Blood was drawn via tail vein before and after treatment to determine clinical grades. Mice were treated daily with 0.01 mg of IL-1ra or placebo for 2 wk. Blood was drawn via tail vein before and after treatment to determine clinical grades.

Radioimmunoassay for mouse muscle AChR (M-AChR) content
α-Bungarotoxin (BTX) (5 × 10⁻⁹ M; Amersham) labeled with [¹²⁵I](0.1 ml), and Triton X-100 solubilized mouse muscle extract (both with and without benzoquinonium (10⁻⁸ M)) were mixed with 10 μl of anti-AChR serum. The resulting complex was precipitated by goat anti-mouse serum and then sedimented by centrifugation, washed in 1 ml of Triton X-100 buffer, and sedimented again. Radioactivity of the pellet was counted in a Packard gamma counter. The AChR precipitated, minus the control value, gave the concentration of M-AChR.

ELISA for anti-M-AChR Ig isotypes
Affinity-purified M-AChR (0.5 μg/ml) was coated onto a 96-well microtiter plate in 0.1 M carbonate bicarbonate buffer (pH 9.6) overnight at 4°C. The plates were blocked with 2% BSA in PBS at room temperature for 30 min. A total of 100 μl of diluted serum samples (to detect IgG1, IgG2b, and IgG2c, 1/2000 dilution; to detect IgM, 1/100 dilution) were added and incubated at 37°C for 90 min. After four washes, HRP-conjugated anti-mouse IgM, IgG1, and IgG2b (Caltag Laboratories) and biotin-conjugated anti-mouse IgG2c (BD Pharmingen), diluted 1/1000 in PBS 0.05% Tween 20 were added and incubated at 37°C for 90 min. Subsequently, 0.3 mg/ml ABTS (diammonium) substrate solution was added to the IgM, IgG1, and IgG2b plates and 100 μl of avidin-labeled peroxidase (Sigma-Aldrich) at 2.5 μg/ml was added to the IgG2c plate and incubated for 30 min. Subsequently, the peroxidase indicator substrate ABTS, in 0.1 M citric buffer, pH 4.35, was added in the presence of H₂O₂, and the mixture was allowed to develop at room temperature in the dark. Plates were read at a wavelength of 405 nm. Serially diluted anti-T-AChR sera were used as a positive control, and normal mouse serum (collected from mice before T-AChR immunization) was used for background determination. The results are expressed as ΔOD (OD of sera after AChR immunization minus OD of sera before AChR immunization) (36, 37).

Cell culture
Draining lymph nodes were harvested and single cell suspensions were prepared. A total of 4 × 10⁶ cells/well were seeded into flat-bottom 96-well plates in RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES buffer, 2-ME (3 × 10 μM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete medium), and stimulated with T-AChR (2.5 μg/ml) or dominant peptide α146–162 (20 μg/ml). Cells were incubated at 37°C in 5% CO₂ for 4–5 days with the addition of [³H]thymidine for the final 16 h of culture. For measurement of cytokine production, cells at a concentration of 4 × 10⁶/ml were seeded into 48-well plates in complete medium with 2.5 μg/ml T-AChR or 20 μg/ml dominant peptide α146–162.
Supernatants were collected at different time points and stored at -20°C until analyzed (37).

Cytokine detection

IFN-γ, IL-1β, IL-2, IL-10, IL-6, and TNF-α in the culture supernatant or sera were measured by ELISA. ELISA plates (Immuno 2; Dynatech) were coated with 2 μg/ml (50 μl/well) anti-IFN-γ, IL-2, IL-10, IL-6, and TNF-α mAb (BD Pharmingen) in 0.1 M carbonate buffer (pH 8.2) overnight at 4°C. The plates were blocked with 200 μl of 10% FBS in PBS for 2 h. Supernatant (100 μl) or 1 μl of sera in 99-μl PBS was added and incubated overnight at 4°C. After the plates were washed four times with PBS and Tween 20 (0.05%), 100 μl of biotinylated anti-cytokine-detecting mAbs (directed to different determinant than the first Ab used to coat ELISA plates) at 1 μg/ml in PBS and 10% FBS were added for 45 min at room temperature. Then 100 μl of avidin-peroxidase (2.5 μg/ml) was added and incubated for 30 min. Subsequently, the peroxidase substrate ABTS in 0.1 M citric buffer pH 4.35 in presence of H2O2 was added and the absorbance was measured at 405 nm (37).

Measurement of serum C3, C1q, C1q-, or C3-conjugated circulating immune complex (CIC) levels by ELISA

The 96-well microtiter plates (Dynex-Immulon 2; Dynatech Laboratories) were coated with goat Abs to mouse C3 (ICN Biomedicals/Cappel) or goat Abs to human C1q (Sigma-Aldrich) in 0.1 M sodium carbonate buffer (pH 8.2) overnight at 4°C. The plates were then blocked with 2% BSA in PBS at room temperature for 30 min. Diluted (1/10 in PBS-0.05% Tween 20) serum samples (30 μl) were added and incubated at 37°C for 90 min. After four washes, HRP-conjugated goat anti-mouse C3 complement (ICN Biomedicals/Cappel) or biotin-conjugated goat anti-human C1q complement (Cedarlane Laboratories), diluted 1/500 in PBS/0.05% Tween 20, was added and incubated at 37°C for 90 min. For C3, ABTS substrate solution in 0.1 M citric buffer (pH 4.3) in the presence of H2O2 was added subsequently, and color was allowed to develop at room temperature in the dark. For C4, 100 μl of avidin-labeled peroxidase (Sigma-Aldrich) at 2.5 μg/ml was added to the plate and incubated for 30 min and then ABTS solution (pH 4.3) was added. Plates were read at a wavelength of 405 nm using a Dynatech ELISA reader, and the results were expressed as OD values. Serum C1q- or C3-CIC levels were determined with the same principle, with the difference that the plate was covered with goat Abs to mouse C3 or goat Abs to human C1q and HRP-conjugated goat anti-mouse IgG (Caltag Laboratories) was used as a secondary Ab.

Results

IL-1ra treatment suppressed the development of clinical EAMG

In experiment 1, two of five (40%) of 0.01 mg IL-1ra-injected mice and two of four (50%) of 0.001 mg IL-1ra-injected mice, compared with six of nine (67%) of placebo injected mice developed clinical EAMG. In experiment 2, the more effective 0.01 mg of IL-1ra injection was tested in a larger group of mice. The incidence of EAMG in 0.01 mg of IL-1ra-injected mice was 4/10 (40%) compared with 9/10 (90%) in placebo control group. The kinetics of the incidence and mean severity of clinical EAMG are illustrated in Fig. 1. In both experiments, IL-1ra-injected mice had a delayed onset, lower total incidence, and less severe clinical EAMG compared with placebo injected group mice (p < 0.05). Thus, when injected multiple times a day, 0.01 mg of IL-1ra suppressed the development of clinical EAMG.

IL-1ra injections led to suppressed serum anti-AChR IgG1 Ab and did not affect total IgG in T-AChR immunized mice

Serum samples from individual mice were collected on days 14 and 60 following T-AChR immunization, and were evaluated for anti-M-AChR Ab concentration by an α-BTX radioimmunoassay and the anti-M-AChR IgG isotypes by ELISA (Fig. 2). Compared with placebo injected mice, IL-1ra-injected mice had lower concentrations of serum anti-M-AChR Ab, especially the anti-AChR IgG1 isotype (p < 0.05, Fig. 2). However, IL-1ra treatment did not influence serum levels of total IgG (Fig. 2). Therefore, IL-1ra injection specifically suppressed anti-AChR IgG1 Ab production, and thus could have contributed to the suppression of clinical EAMG.

IL-1ra injections suppressed serum C3 production in T-AChR-immunized mice

Sera from individual mice collected on days 14 and 60 following T-AChR immunizations were measured for C1q, C3, C1q-CIC, and C3-CIC by ELISA. Compared with placebo injected mice, IL-1ra-injected mice had lower serum concentrations of C3 (p < 0.05, Fig. 3). IL-1ra injections did not influence serum levels of C1q, C1q-CIC, and C3-CIC. C3 activation is pivotal for membrane attack complex (MAC) formation, anti-AChR Ab production, and EAMG induction (5, 8). C3 gene knockout (KO) mice are highly resistant to EAMG induction and this resistance is associated with low levels of serum anti-AChR Ab and absence of MAC deposits at the NMJ (5). Therefore, reduction in serum C3 levels following
IL-1ra injection could have contributed to reduced anti-AChR Ab responses, reduced NMJ MAC formation and thus suppression of clinical EAMG.

**IL-1ra injections led to reduced levels of proinflammatory cytokines in serum samples of T-AChR immunized mice**

After the boost with T-AChR (day 60), a significant number of placebo injected mice developed clinical EAMG, with associated increases in serum levels of IL-1β, IL-2, IL-6, IFN-γ, and TNF-α (Fig. 4). On the contrary, the reduced clinical incidence of EAMG in IL-1ra-injected (0.01 mg) mice was associated with reduced serum levels of IL-1β, IL-2, IL-6, IFN-γ, and TNF-α (p < 0.05) (Fig. 4). Serum IL-10 levels were relatively unchanged by IL-1ra injections. These results are in consistent with the previously published reports revealing IL-1 as an important activator of various cytokines (25, 26). They also indirectly prove that the cytokine production and activating effects of IL-1 is effectively diminished by our IL-1ra treatment protocol.

**IL-1ra injection led to reduced cellular immune responses to T-AChR and its dominant peptide α146–162**

To test the biological modulatory effect of IL-1ra in the cellular immune responses to AChR and its dominant peptide, LNC from T-AChR immunized mice were stimulated in vitro with T-AChR or α146-162 peptide, at the termination of the experiment (60 days after first AChR immunization). LNC of IL-1ra-injected mice demonstrated a reduced proliferative response to T-AChR and α146–162 peptide, compared with those of placebo injected mice (Fig. 5). Lymph node production of AChR and α146–162 peptidespecific IL-2, and TNF-α were significantly (p < 0.05) suppressed in IL-1ra-injected mice compared with placebo injected mice. Additionally, AChR, but not α146–162 peptide stimulated LNC produced lower amounts of IL-6 and IFN-γ as compared with placebo injected mice. Even ex vivo, IL-1ra failed to influence the AChR-specific production of IL-10 by LNC. The data implicate that the IL-1ra-mediated suppression of clinical disease was associated with the suppression of AChR- and/or immunodominant peptide-specific lymphocyte proliferation, IL-2, IL-6, IFN-γ, and TNF-α production. The reduction in anti-AChR IgG1 Ab production following IL-1ra injection could be due to the reduction in the production of IL-2 and IL-6 because these cytokines are required for an optimal IgG1 response.

**IL-1ra treatment in mice with ongoing clinical EAMG suppressed anti-AChR IgG1 and reduced the clinical severity of disease**

For treatment study, 24 of 60 adult C57BL/6 male mice that developed clinical EAMG grades at or higher than 1.5 after two or three immunizations with T-AChR in CFA were divided into two groups of 12 mice, each with equivalent grades. Mice with clinical EAMG were injected s.c. five times (daily), for 2 wk, with 0.01 mg of IL-1ra or placebo. A significant improvement (p < 0.05) in clinical MG (reduction in clinical grade) was observed 4 wk following the initiation of IL-1ra treatment compared with placebo treatment (Fig. 6A). Ten of 12 (83%) IL-1ra-treated mice showed clinical improvement. One mouse did not show any change in its clinical condition, whereas the muscle weakness of another mouse deteriorated. On the contrary, the clinical condition of 5 of 12 (41.7%) placebo injected mice worsened, and 7 of 12 (58.3%) had no change.

The primary pathology in EAMG is a significant reduction of muscle AChR due to Ab- and complement-mediated attack of the NMJ (4, 5). The numbers of α-BTX binding sites in the muscle, which reflect the amount of functionally available muscle AChR, were measured in IL-1ra and placebo-injected mice. The functional AChR (α-BTX binding sites) level in IL-1ra-injected mice was significantly higher (p < 0.05) than that of placebo injected mice (Fig. 6B). Therefore, IL-1ra administration reduced AChR loss in the NMJ of mice immunized with T-AChR, thus contributing to clinical improvement.

Sera from individual mice were collected before and after IL-1ra or placebo treatment, and anti-M-AChR Ab concentration was evaluated by α-BTX radioimmunoassay (Fig. 6C) and the anti-M-AChR IgG isotypes were measured by ELISA (Fig. 7). Compared with placebo injected mice, IL-1ra-injected mice had reduced concentration of serum anti-M-AChR Ab in the radioimmunoassay. IL-1ra treatment suppressed predominantly anti-AChR IgG1 isotype (p < 0.05) and failed to influence anti-AChR IgG2b and IgG2c isotypes. Thus the clinical improvement following IL-1ra treatment in mice with ongoing clinical EAMG was also associated with reduced production of serum anti-AChR IgG1 isotype.

**Discussion**

A cytokine hierarchy exists in the development of EAMG. IL-6, IL-18, IL-1, and TNF are very critical for activation of AChR-specific T and B cells (8, 13, 16, 17, 19). IL-12, IFN-γ, IL-10, and
IL-5 also contribute to the development of EAMG (9–11, 14, 20), whereas IL-4 does not play a significant role (38). IL-1β gene KO mice have been shown to be resistant to EAMG (19). This resistance was associated with reduced serum anti-AChR Ab levels, diminished lymphocyte proliferation capacity in response to AChR stimulation and decreased LNC IL-2, IL-4, and IFN-γ production (19). In our experiments, we obtained very similar results in IL-1ra-treated mice (reduced serum anti-AChR Ab levels, decreased lymphocyte proliferation and reduced LNC IL-2 and IFN-γ production). Additionally, we demonstrated that IL-1ra treatment is also reducing serum anti-AChR IgG1 levels, serum cytokine and C3 levels and LNC IL-6 and TNF-α production. The IL-1β gene KO mice might have other constitutional defects due to IL-1 increased levels of TNF-inflammatory functions. However, in this study, we observed disease mechanism should not have been plausibly eliminated in IL-1ra-treated mice. The reduction of anti-AChR IgG1 following IL-1ra treatment were associated with the reduction of serum anti-AChR IgG1 levels. One of the main mechanisms of IL-1ra treatment could have culminated in the reduction of serum anti-AChR IgG1 Abs and muscle AChR loss, thus improving the clinical status of myasthenic mice (Fig. 8).

Our studies suggest that inhibition of IL-1 in EAMG with IL-1 inhibitors may limit the autoimmune response to AChR. Plausibly, IL-1 inhibitors applied in combination with other anti-proinflammatory cytokines might be more beneficial in the treatment of MG. Therefore, the combined therapeutic effect of recombinant IL-1ra treatment could have culminated in the reduction of serum anti-AChR IgG1 Abs and muscle AChR loss, thus improving the clinical status of myasthenic mice (Fig. 8).

Disclosures
The authors have no financial conflict of interest.

References


