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J Immunol 2003; 171:2769-2772; ;
doi: 10.4049/jimmunol.171.6.2769
http://www.jimmunol.org/content/171/6/2769

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Requirement for Growth Hormone-Releasing Hormone in the Development of Experimental Autoimmune Encephalomyelitis[1]

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***Growth hormone (GH)-releasing hormone (GHRH) is a neuropeptide that stimulates secretion of GH from the pituitary gland. Although GHRH and its receptor (GHRHR) are expressed in leukocytes, physiological function of GHRH in the immune system remains unclear. To study the influence of GHRH in autoimmunity, susceptibility to experimental autoimmune encephalomyelitis (EAE) was examined in C57BL/6J-*Ghrhr$^{lit/lit}$ *(*lit/lit*), mice deficient in the GHRHR gene. We found that* lit/lit *mice were resistant to myelin oligodendrocyte glycoprotein (MOG)-induced EAE. Splenocytes from MOG-immunized* lit/lit *mice proliferated normally in response to MOG peptide, suggesting that activation of MOG-specific T cells in GHRHR-deficient mice is not impaired. Our data strongly suggest that GHRH plays a crucial role in the development of EAE and may provide the basis for a novel therapeutic approach protecting from autoimmune diseases. The Journal of Immunology,* 2003, 171: 2769–2772.**

Experimental autoimmune encephalomyelitis (EAE)[3] is a T cell-mediated autoimmune disease that is the most common animal model for human multiple sclerosis (MS). Both EAE and MS are characterized clinically by chronic or relapsing paralysis and histologically by inflammatory infiltrates in the CNS and demyelination (1–5). EAE can be induced in susceptible strains of mice by immunization of specific myelin Ags such as myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein. Progression of EAE is regulated at many points during the course of disease, including activation of autoreactive T cells, transmigration of the cells across the blood-brain barrier, recruitment of inflammatory cells to the CNS, establishment and maintenance of an autoimmune response, and inflammation in the CNS.

Growth hormone (GH)-releasing hormone (GHRH) is a hypothalamic neuropeptide that stimulates secretion of GH from the pituitary gland. GHRH receptor (GHRHR), expressed on pituitary somatotrope cells, is a member of the family of G protein-coupled receptors and mediates the action of GHRH in stimulating growth hormone synthesis and secretion (6, 7). The most striking evidence for importance of GHRH in GH secretion is provided by a spontaneous mutant strain of mice. The "*little*" is a missense mutation in the GHRHR gene, that disrupts the receptor function (8, 9). The mice homozygous for this mutation, C57BL/6J-*Ghrhr$^{lit/lit}$* (*lit/lit*) shows reduced GH secretion and dwarf phenotype (8, 9). Because GHRH and GHRHR have been found in leukocytes, it is suggested that GHRH is involved in the immune system (10–13). In vitro studies demonstrated the direct stimulatory and inhibitory effect of GHRH on immune cells (14, 15). However, the physiological role of GHRH in immune function is still unclear.

In this study, we investigated the contribution of GHRH to the pathogenesis of EAE. We report that upon immunization with immunodominant $MOG_{35–55}$ peptide, GHRHR-deficient *lit/lit* mice did not develop any neurological impairment. Splenocytes from MOG-immunized *lit/lit* mice proliferated normally in response to MOG peptide, whereas anti-MOG IgG was increased in *lit/lit* mice. Our data presented here suggest that GHRH is playing a critical role in the development of EAE in mice and raise a possibility that the GHRH antagonist is beneficial in the treatment of MS.

Materials and Methods

Animal experimentation

Animal experiments in this study were performed according to the guidelines of the Animal Studies Committee of the Research Division of Sumitomo Pharmaceuticals. GHRHR-deficient C57BL/6J-*Ghrhr$^{lit/lit}$* mice (*lit/lit*), their heterozygous littermates (*lit/+*), control C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were female, 6 wk of age at the time of immunization, and were bred at the Animal Facility (Research Division, Sumitomo Pharmaceuticals) under pathogen-free conditions.

Ags and reagents

The immunodominant $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVH LYRNGK) was synthesized and purified through HPLC by Qiagen (Tokyo, Japan). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA).

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Received for publication May 30, 2003. Accepted for publication July 11, 2003.

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[1] These studies were supported by Sumitomo Pharmaceuticals.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; GH, growth hormone; GHRH, GH-releasing hormone; GHRHR, GH-releasing hormone receptor; CRH, corticotropin-releasing hormone.

0022-1767/03/$02.00

Induction and evaluation of EAE

The *lit/lit*, *lit/+*, and control C57BL/6J mice were immunized s.c. with 100 μl of an emulsion composed of $MOG_{35–55}$ peptide (200 μg/mouse) in saline and an equal volume of CFA containing *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Mice received 200 ng of pertussis toxin (List Biological Laboratories) in 0.1 ml of saline (i.p.) on the day of and 2 days following immunization. Mice were examined every day for signs of EAE. The clinical severity was graded into six categories (grade 0, no sign; grade 1, tail paralysis; grade 2, mild hind limb weakness; grade 3, moderate to severe hind limb paresis and/or mild forelimb weakness; grade 4, complete hind limb paralysis and/or moderate to severe forelimb weakness; grade 5, quadriplegia or moribund; grade 6, death).

Analysis of proliferative response and cytokine production

Spleens were obtained from *lit/lit, lit/+*, and C57BL/6J mice 32 days after immunization with MOG peptide. Single cell suspension was prepared, and cultured in round-bottom 96-well microtiter plates (Asahi Techno Glass, Tokyo, Japan) at a density of 5×10^5 viable cells/well in a total volume of 200 μl of RPMI 1640 medium (Life Technologies, Gaithersburg, MD), supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine (Life Technologies), 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 100 U/ml gentamicin (Life Technologies). Cells were cultured at 37°C in 100% humidity and 5% CO_2 in the presence or absence of varying concentrations of $MOG_{35–55}$ peptide or 1 μg/ml Con A (Sigma-Aldrich). For proliferation assay, 1 μCi [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added to each culture at 72 h, and cells were harvested 16 h later (Wallac, Gaithersburg, MD). Radioactivity was counted in a beta scintillation counter. For cytokine assay, cells were cultured by the same protocol for proliferation assay and the supernatants were harvested 48 h after initiation of cultures.

Measurement of serum anti-MOG IgG

Sera were collected 30 days after immunization with $MOG_{35–55}$ peptide. Anti-$MOG_{35–55}$ peptide IgG levels in sera were measured by ELISA. MaxiSorp 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 100 μl of 10 μg/ml $MOG_{35–55}$ peptides in 0.05 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. The wells were incubated with PBS-1% BSA for 2 h at room temperature. After washing, a 100-μl aliquot of each mouse serum diluted 50-fold in PBS-1% Tween 20 was applied and incubated for 2 h at room temperature. After another washing, 100 μl of goat anti-mouse IgG (whole molecule) Abs conjugated to HRP (Sigma-Aldrich) diluted 1/1000 in PBS-1% Tween 20 were added and incubated for 2 h at room temperature. The plates were washed three times and color development of *p*-nitrophenylphosphate (Sigma-Aldrich) was monitored at 405 nm in an ELISA plate reader.

Results

GHRHR-deficient mice were resistant to the induction of EAE

To address the potential involvement of GHRH in the pathogenesis of EAE, we determined whether EAE can be induced in GHRHR-deficient *lit/lit* mice. EAE was induced with the synthetic MOG peptide, $MOG_{35–55}$, which is encephalitogenic in C57BL/6J (H-2^b) mice (16). The results shown in Fig. 1 demonstrate that most of the control C57BL/6J mice readily developed EAE. In contrast, no *lit/lit* mice developed any clinical symptoms of EAE during the entire periods of observation (32 days). The disease severity of individual animals is shown in Fig. 1*B*. Maximal scores of control C57BL/6J mice were significantly ($p < 0.005$) different from those of *lit/lit* mice. The heterozygous *lit/+* mice, which do not show dwarf phenotype, developed milder disease compared with control C57BL/6J mice. These results indicate that GHRH is required for the development of EAE.

MOG-specific immune responses in GHRHR-deficient mice were not impaired

Resistance to EAE in *lit/lit* mice can be due to the reduction of immune response to the $MOG_{35–55}$ peptide used for EAE induction. To address this issue, we examined whether activation of myelin-specific T cells was normal in *lit/lit* mice. Splenocytes were collected from $MOG_{35–55}$ peptide-immunized *lit/lit*, *lit/+*, and control C57BL/6J mice 32 days after immunization

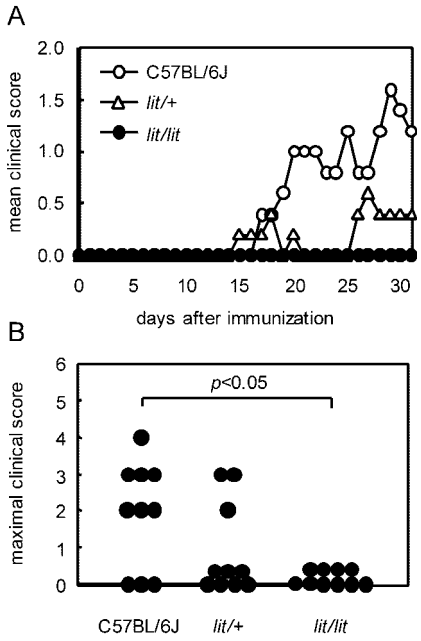

FIGURE 1. GHRHR-deficient *lit/lit* mice are resistant to MOG-induced EAE. EAE was induced in the mice by immunization with $MOG_{35–55}$ peptide in CFA. *A*, Clinical scores of mice in respective groups ($n = 5$ mice per group) were averaged and are shown for every day. These data are representative of two independent experiments. *B*, Maximal clinical scores of individual mice. The averages were 0.0 ± 0.0, 0.8 ± 1.3, and 1.9 ± 1.4 for control C57BL/6J, *lit/+*, and *lit/lit* mice, respectively. These data are pooled from two independent experiments.

and tested in vitro for their proliferation in response to $MOG_{35–55}$ peptide. As shown in Fig. 2*A*, splenocytes from *lit/lit* and *lit/+* mice proliferated normally in response to MOG peptide. Moreover, Con A-induced proliferation was also intact in splenocytes prepared from *lit/lit* mice (Fig. 2*B*). These results suggest that GHRHR deficiency did not affect the activation of MOG-specific T cells.

Because Abs to the MOG Ag may contribute to demyelinating lesions and to the severity of EAE (17), we examined the level of anti-$MOG_{35–55}$ Ab in the serum of MOG-immunized *lit/lit* mice. As shown in Fig. 3, the level of anti-$MOG_{35–55}$ IgG

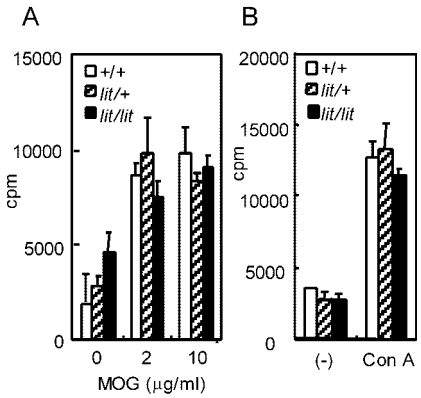

FIGURE 2. MOG-specific proliferation of lymphocytes in vitro was not impaired in GHRHR-deficient *lit/lit* mice. EAE was induced in the mice as in Fig. 1 and sacrificed 32 days after immunization. Splenocytes were cultured with various concentrations of $MOG_{35–55}$ peptide (*A*) or Con A (*B*). Proliferation was measured by incorporation of [^{3}H]thymidine. Results are shown as means ± SD. The experiments were repeated twice with similar results.

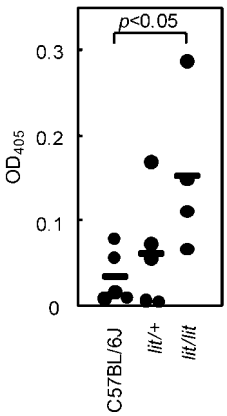

FIGURE 3. Induction of $MOG_{35–55}$-specific Abs in GHRHR-deficient *lit/lit* mice. Serum samples were prepared from peripheral blood collected at 30 days after EAE induction. Each sample was diluted 50-fold and tested for anti-$MOG_{35–55}$ IgG by ELISA using plate coated with $MOG_{35–55}$ peptides and HRP-conjugated anti-IgG Ab. Horizontal bars represent the mean values of each group.

in serum was higher in *lit/lit* mice compared with that in wild-type mice. Thus, it is unlikely that resistance of GHRHR-deficient mice to MOG-induced EAE was due to a deficient humoral immune response.

Discussion

In the present study, we demonstrated that GHRH is crucial for the induction of EAE. None of GHRHR-deficient *lit/lit* mice developed any sign of clinically evident disease after active immunization with $MOG_{35–55}$ peptide. Interestingly, heterozygous *lit/+* mice developed mild disease symptoms compared with control C57BL/6J mice, suggesting a dose-related effect of GHRH. Moreover, because *lit/+* mice do not show dwarf phenotype, it is unlikely that the resistance of *lit/lit* mice to EAE induction is a secondary phenomenon experienced by dwarf phenotype. Taken together, these results suggest that GHRH plays a critical role in the pathogenesis of EAE.

GHRH stimulates GH secretion from the pituitary gland. GH, directly or via induction of insulin-like growth factor-I, is implicated in lymphocyte development and function (18, 19). Earlier observations of GH-deficient mice also suggested a pivotal role of GH in the immune system, because Ames (*df/df*) and Snell-Bagg (*dw/dw*) dwarf mice show severe immune deficits (20). Because *lit/lit* mice also show reduced GH secretion and dwarf phenotype, it is conceivable that EAE resistance of *lit/lit* mice is caused by insufficient immune function. To address this question, we analyzed the immune response of $MOG_{35–55}$-immunized *lit/lit* mice. However, the in vitro proliferative response to immunized $MOG_{35–55}$ peptide was not impaired. Con A-induced proliferation was also normal as compared with control C57BL/6J mice. These results suggest that EAE resistance of *lit/lit* mice was not due to insufficient T cell response. Serum anti-$MOG_{35–55}$ IgG levels were increased in *lit/lit* mice. Therefore, although the Ab response by the B cell is also involved in the development of EAE (21, 22), it is unlikely that EAE resistance of *lit/lit* mice is due to a decreased B cell response to immunized $MOG_{35–55}$ peptide. Taken together, these observations suggest that the resistance of *lit/lit* mice to EAE is not due to the reduced immune response caused by insufficient GH secretion. However, the data presented here do not exclude a possible disease-modifying effect of MOG-specific autoantibodies in EAE. Whether the higher level of $MOG_{35–55}$-specific Abs is involved in EAE resistance of *lit/lit* mice remains to be clarified.

The neuroendocrine system has potent effects on the immune system. These two systems can communicate in a bidirectional fashion using a common set of signal molecules and their receptors (23). For example, corticotropin-releasing hormone (CRH), a hypothalamic key regulator of stress response in the hypothalamic-pituitary-adrenal axis, suppressed EAE not only mediated by the hypothalamic-pituitary-adrenal axis, but also by direct effects on the immune system (24). In addition, the proinflammatory effects of CRH in vivo and in vitro have been also reported (25, 26). CRH and its receptor are widely distributed outside the brain including immune cells (27–31). The immune cells can also produce over 20 different neuropeptides such as adrenocorticotropic hormone, which is successful in reducing the intensity and duration of relapses of MS (32, 33). In addition, large scale analysis of gene transcripts in MS lesions revealed that leptin, the melanocortin 4 receptor, and the adrenocorticotropic hormone receptor are elevated at the site of inflammation in the brain (34). These data suggest that the bidirectional interaction between immune and neuroendocrine systems may critically influence the susceptibility of autoimmune diseases.

The expression of GHRH and GHRHR on lymphocytes was previously reported (10–13). In addition, in vitro studies demonstrated that GHRH stimulates proliferation of lymphocytes and inhibits NK cell activity (14, 15) and chemotaxis (35) of human lymphocytes. These data suggest direct involvement of GHRH in immune system. However, the precise roles of GHRH in the regulation of the immune system in vivo are unclear. Although our data presented here suggest the requirement of GHRH in EAE development, the mechanisms by which GHRH is involved in autoimmunity remains unknown. Further investigation of the development of chronic inflammatory disorders regarding the influences of the neuroendocrine system including GHRH-GH axis may provide a clue for understanding the etiology of autoimmune diseases.

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