Low-Dose Estrogen Therapy Ameliorates Experimental Autoimmune Encephalomyelitis in Two Different Inbred Mouse Strains

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Low-Dose Estrogen Therapy Ameliorates Experimental Autoimmune Encephalomyelitis in Two Different Inbred Mouse Strains

Bruce F. Bebo, Jr., Amber Fyfe-Johnson, Kirsten Adlard, Aaron G. Beam, Arthur A. Vandenbark, and Halina Offner

It has been proposed that homeostatic levels of estrogen can enhance female susceptibility to autoimmunity, whereas the heightened levels of estrogen associated with pregnancy are protective. This hypothesis was tested using the mouse model of experimental autoimmune encephalomyelitis (EAE). Diestrous (<100 pg/ml in serum) levels of 17β-estradiol were found to significantly reduce the clinical manifestations of active EAE in both male and female mice. Estriol was also effective but at doses below those previously established for pregnancy. The reduction in disease severity was accompanied by a coincident reduction in the number and size of inflammatory foci in the CNS of estrogen (17β-estradiol or estriol)-treated mice. Recipients of encephalitogenic T cells from low-dose estrogen-treated mice developed less severe paralysis than mice receiving T cells from placebo-treated mice. A modest shift in Th1/Th2 balance suggested that low dose estrogen therapy could bias the immune reaction toward a protective anti-inflammatory cytokine response. However, estrogen treatment at the onset of active EAE failed to reduce disease severity, a result that is consistent with the hypothesis that naïve cells are more sensitive to sex hormones than differentiated effector cells. These data suggest that treatment with low doses of estrogen can reduce the capacity of developing myelin-reactive T cells to initiate disease and challenges the idea that increased susceptibility to autoimmunity in females is dependent on homeostatic levels of estrogen. The Journal of Immunology, 2001, 166: 2080–2089.
increased Th1 immunity and susceptibility to cell-mediated auto-immune diseases, whereas high levels shift the response toward Th2 immunity and protection from cell-mediated pathology (23). This rationale explains both the increase in the incidence and severity of EAE in females and the protection afforded by increased estrogen levels during pregnancy. However, the concept of biphasic responses to estrogen remains a hypothesis and has yet to be critically tested in the EAE model.

In this study, the efficacy of low-dose estrogen therapy was evaluated in two different models of EAE. Treatment with low levels of both 17β-estradiol (E2) and E3 reduced the severity of EAE caused by active immunization. Estrogen treatment was equally effective in both SJL and B10.PL models of disease. No differences in the level of protection were observed between E2 and E3, and both genders were equally sensitive to the effects of estrogen. Low-dose estrogen treatment of donor mice reduced the severity of EAE induced by the adoptive transfer of PLP<sub>139-151</sub>-specific T cells, but treatment of EAE at disease onset had no effect, suggesting that the priming phase of the immune response was more sensitive to estrogen regulation. No evidence was found for a biphasic response to estrogen in EAE. These observations challenge the hypothesis that increased susceptibility to autoimmune disease in females is dependent on low levels of estrogen found during estrus or diestrus.

Materials and Methods

Animals

Age-matched SJL/J and B10.PL mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Young adult (≤10 wk old) mice were used for all experiments mentioned in this report. The animals were housed in the Animal Resource Facility at the Portland Veterans Affairs Medical Center in accordance with institutional guidelines.

Antigens

Mouse PLP<sub>139-151</sub> (HCLGKWLHDPDKF) and myelin basic protein (MBP) peptide Ac1-11 (ASQKRPSQRK) were synthesized using solid-phase chemistry on a Synergy 432A peptide synthesizer (Applied Biosystems, Foster City, CA), and purified prior to use.

Estrogen treatment and measurement of serum estrogen levels

Sixty-day release pellets of E2, E3, and placebo were implanted i.c. in the scapular region behind the neck using a 12-gauge trochar as described by the manufacturer (Innovative Research, Sarasota, FL). The mice were implanted 1 wk before immunization with the appropriate myelin Ag.

Representative animals were bled by cardiac puncture, and the blood was allowed to clot at 4°C overnight. The samples were centrifuged, and the sera were collected and stored at −80°C until hormone analysis was performed. Serum levels of E2 and E3 were determined by radioimmunoassay (RIA) after Sephadex LH-20 column chromatography. All samples were analyzed in a single assay for each hormone.

Induction of EAE

SJL mice were inoculated s.c. in the flanks with 0.2 ml of an emulsion containing 150 μg of PLP<sub>139-151</sub> in saline and an equal volume of CFA containing 200 μg of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). B10.PL mice were immunized with an emulsion containing 400 μg of MBP Ac1-11 and 200 μg of Mycobacterium tuberculosis. Disease induction in B10.PL mice required treatment with pertussis toxin on the day of immunization (75 ng/mouse) and 2 days later (200 ng/mouse). For adoptive transfer, SJL mice were implanted with either E2 or E3 pellets and, 1 wk later, immunized as described above. Ten days after the immunization, splenocytes were recovered and stimulated in vitro with 10 μg of PLP<sub>139-151</sub> for 72 h. Live cells were isolated by ficoll density centrifugation, and 30 × 10<sup>6</sup> T cell blasts were injected i.p into naive female SJL hosts. The mice were examined daily for clinical signs of disease and scored according to the following scale: 0, normal; 1, minimal or mild hind limb weakness; 2, moderate hind limb weakness or mild ataxia; 3, moderately severe hind limb weakness; 4, severe hind limb weakness or moderate ataxia; 5, paraplegia with no more than moderate forelimb weakness; 6, paraplegia with severe forelimb weakness or severe ataxia.

Histopathology

The intact spinal column was removed from mice during the peak of clinical disease and fixed in 10% phosphate buffered formalin. The spinal cords were dissected after fixation and embedded in paraffin before sectioning. The sections were stained with luxol fast blue-periodic acid schiff-hematoxylin and analyzed by light microscopy. Semiquantitative analysis of inflammation and demyelination was determined by examining at least ten sections from each mouse.

Immunofluorescent staining for flow cytometry

Draining lymph node (DLN) cells were removed during the peak of clinical symptoms and analyzed for the expression of cell surface proteins by fluorescence staining ex vivo. The following fluorochrome-conjugated Abs obtained from PharMingen (San Diego, CA) were used for the direct staining of DLN cells: anti-CD4, anti-CD25, anti-CD69, anti-CD95 ligand (anti-CD95L), anti-CD44, anti-CD62L, anti-CD49d. Two-color immunofluorescent analysis was performed on a FACSScan instrument (Becton Dickinson, Mountain View, CA) using CellQuest software. For each experiment the cells were stained with isotype control Abs to establish background staining and to set the quadrants before calculating the percent positive staining cells.

Proliferation assays

DLN cells were recovered from the mice at peak of clinical EAE (days 12–16 postimmunization) as previously described (13). The in vitro proliferative response was determined using a standard microtiter assay (24). Briefly, DLN cells were cultured in 96-well, flat-bottom tissue culture plates at 4 × 10<sup>4</sup> cells per well in stimulation medium alone (control) or with test Ags (i.e., PLP<sub>139-151</sub>) and incubated for 3 days at 37°C in 7% CO<sub>2</sub>. Wells were pulsed for the final 18 h with 0.5 μCi of [3H]methylthymidine (Amersham, Arlington Heights, IL). The cells were harvested onto glass fiber filters, and tritiated thymidine uptake was measured by a liquid scintillation counter. Results were determined from the means of triplicate cultures. Stimulation indices were determined by calculating the ratio of Ag-specific cpm to control cpm.

Cytokine detection by ELISA

DLN cells were cultured at 4 × 10<sup>5</sup>/ml and stimulated with the appropriate Ag in 24-well culture plates. Cell culture supernatants were recovered between 48 and 72 h and frozen at −70°C until needed for the cytokine assay.

Measurement of cytokines was performed by ELISA using cytokine-specific capture and detection Abs (PharMingen). Standard curves for each assay were generated using recombinant mouse cytokines (PharMingen), and the concentration of cytokines in the cell supernatants was determined by interpolation from the appropriate standard curve. IFN-γ, TNF-α, and IL-12 were chosen as representative Th1 cytokines, whereas IL-4 and IL-10 were measured as representative Th2 cytokines.

PLP<sub>139-151</sub>-specific Ab ELISA

Nunc-Immuno 96-well ELISA plates (Nunc, Roskilde, Denmark) were coated with PLP<sub>139-151</sub> at 4 μg/ml in PBS overnight at 4°C. The plates were washed and blocked before the addition of serum at the indicated dilution in triplicate. The samples were incubated overnight at 4°C, and the plate was washed before the addition of an affinity-purified, biotinylated goat anti-mouse Ig (diluted 1:10,000) detecting Ab (Accurate Chemical and Scientific, Westbury, NY). The plates were incubated for 1 h at room temperature before they were washed. A 1:400 dilution of avidin-peroxidase conjugate (Sigma, St. Louis, MO) was added to each well, and the plates were incubated for an additional 45 min. After the final wash, a peroxidase substrate (3,3′,5,5′-tetramethylbenzidine; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to the wells, and the reaction was stopped by the addition of 0.18 M sulfuric acid. The plates were read in a microtiter’s kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Wells coated with an irrelevant peptide (myelin oligodendrocyte glycoprotein 35-55) acted as a negative control.

Anexin V staining

Female SJL mice were treated with estrogen pellets and immunized with PLP<sub>139-151</sub> as described earlier. Ten days after immunization, DLN and splenocytes were recovered and an aliquot of cells was stained with anti CD4-FITC, annexin V-PE, and 7-aminomethylcoumarin D as per the manufacturer’s protocol (PharMingen). The remaining cells were stimulated with PLP<sub>139-151</sub> for 72 h, washed, and stained as above. CD4+ T cells were gated on acquisition, and three-color immunofluorescent analysis of apoptosis was performed using a FACSScan instrument and CellQuest software.
Results

Low-dose E2 treatment reduced the severity of EAE in SJL mice

It has been previously established that pregnancy can ameliorate cell-mediated autoimmune diseases such as rheumatoid arthritis (25, 26) and MS (5–7). Estrogens and other sex hormones are found at high levels in the serum during pregnancy and are thought to be an important factor mediating this protection. As a result, high-dose estrogen therapy is being considered for the treatment of these diseases in humans. The evaluation of low-dose estrogen therapy as an alternative to high-dose therapy has not been fully investigated. The purpose of this study was to determine whether low doses of estrogen could provide protection against EAE an animal model for MS. Female SJL mice were implanted with 60-day release tablets (Innovative Research, Sarasota, FL) containing E2 1 wk before the active induction of EAE by immunization with PLP139-151. The dose of E2 chosen for these studies (Table I) was intended to mimic the levels of E2 found during pregnancy, estrus, or diestrus phases of the hormone cycle (27) (Table II). E2 levels were measured in representative animals and were determined to be equivalent to those reported by the manufacturer (data not shown). Pregnancy levels of E2 reduced the incidence and severity of clinical disease in a manner similar to that reported previously (Fig. 1). More importantly, low levels of E2 profoundly reduced the clinical manifestations of disease. Pellets releasing as little as 25–50 pg of E2 per ml of serum significantly diminished the severity of paralysis when compared with placebo controls (Fig. 1). Estrus levels of E2 also delayed the onset of clinical disease achieving statistical significance in animals implanted with 0.36 mg E2 pellets. A significant reduction in the incidence of disease was only seen in animals implanted with pregnancy levels of E2. In addition, pathological examination revealed a dramatic reduction in mononuclear cell infiltration and demyelination in the spinal cords of E2-protected mice when compared with placebo-treated mice (Fig. 2).

Low-dose E3 treatment reduced the severity of EAE in SJL mice

The diminution of MS-related symptoms during pregnancy appear to be most profound during the third trimester (7). E3 is a hormone produced by the placenta and is at its highest levels during the third trimester of pregnancy. It is a hormone that is involved in the regulation of the menstrual cycle, pregnancy, and lactation.

Table I. Serum concentration of E2 and E3 expected over 60 days by implantation of hormone pellets

<table>
<thead>
<tr>
<th>Pellet (mg)</th>
<th>E2 (pg/ml)</th>
<th>E3 (pg/ml)</th>
</tr>
</thead>
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<tr>
<td>15</td>
<td>9,000–10,000</td>
<td>10,000–15,000</td>
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<td>5</td>
<td>3,000–4,000</td>
<td>5,000–6,000</td>
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<tr>
<td>2.5</td>
<td>1,500–2,000</td>
<td>2,000–3,000</td>
</tr>
<tr>
<td>1.5</td>
<td>800–1,000</td>
<td>800–1,000</td>
</tr>
<tr>
<td>0.36</td>
<td>150–200</td>
<td>150–200</td>
</tr>
<tr>
<td>0.1</td>
<td>25–50</td>
<td>40–50</td>
</tr>
<tr>
<td>0.025</td>
<td>5–10</td>
<td>10–20</td>
</tr>
</tbody>
</table>

Table II. Serum concentration of E2 and E3 expected for mouse serum

<table>
<thead>
<tr>
<th></th>
<th>E2 (pg/ml)</th>
<th>E3 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diestrus</td>
<td>20–30</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Estrus</td>
<td>100–200</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>5,000–10,000</td>
<td>2000–3000</td>
</tr>
</tbody>
</table>
trimester. Recently, high-dose E3 treatment has been shown to reduce the severity of EAE induced by the adoptive transfer of myelin-reactive T cells (20). In this study, high-dose E3 therapy effectively reduced the incidence and severity of EAE induced by active immunization of female SJL mice with PLP139-151 (Fig. 3). Treatment of female mice with 1.5 mg E3 pellets resulted in serum hormone levels that were one-half to one-third of that known to result from pregnancy (Table I). Disease severity was significantly reduced in mice receiving low-dose E3 treatment as reflected by a significant decrease in mean peak disease score and cumulative disease index (CDI; Fig. 3). The diminution in clinical disease score was accompanied by a substantial reduction in inflammation and demyelination upon pathological examination (Fig. 2). The direct comparison of E2 and E3 in the same animal model also allowed us to determine whether one form of estrogen was more or less potent than the other form. No statistically significant differences (as determined by the Fisher exact test) in the incidence or severity of EAE were found, indicating that E2 and E3 were equally protective.

Low-dose estrogen therapy reduced the severity of EAE in B10.PL mice

The sensitivity of the immune response to estrogens may be linked to genetic factors both within and outside of the immune response loci. B10.PL mice are genetically distinct from SJL mice and respond to a different dominant myelin Ag, MBP Ac1-11. The sensitivity of these mice to estrogen therapy was tested by treating the mice with estrogen-containing pellets before immunization. Pregnancy levels of E2 profoundly lowered the incidence, delayed the onset, and reduced clinical paralysis (Table III). Low-level E2 treatment also significantly delayed the onset and reduced the severity of EAE induced by the immunization with MBP Ac1-11 peptide (Table III). When the cumulative disease indices and peak disease scores were compared (Fisher’s exact test), no significant differences in E2 sensitivity between SJL and B10.PL mice at low E2 levels were found (data not shown); however, B10.PL mice appeared to be more sensitive to high-dose E2 treatment. Strain differences in peak disease score and CDI were significant in mice receiving 2.5 mg E2 pellets ($p < 0.005$). E3 also delayed the onset and reduced the severity of disease in B10.PL mice (Table IV), but no differences in sensitivity to E3 were detected between these mice and SJL mice as determined by the Fisher exact test (data not shown).

**Male SJL mice are sensitive to estrogen**

The direct influence of estrogen on cells of the immune system is supported by evidence demonstrating estrogen receptors (ERs) in immunocompetent cells (28, 29). Furthermore, we have found that ER expression is not limited to female cells, but is also present in male cells (B. F. Bebo and H. Offner, unpublished observations). Because male cells are potentially sensitive to estrogens, estrogen therapy was performed on male SJL mice. Male mice were treated with E2- and E3-containing pellets as described previously and 1 wk later they were immunized with PLP139-151. Treatment with either E2 or E3 delayed the onset and reduced the severity of clinical disease, even at doses equivalent to estrus levels (150–200 pg/ml) in females (Fig. 4). No significant differences in estrogen sensitivity (as determined by the Fisher exact test) were detected between males and females (data not shown).
Low-dose estrogen therapy reduces the capacity of T cells to transfer EAE

Estrogen has been shown to directly regulate immunocompetent cell function by several investigators (29, 30). However, it is unclear whether estrogen influences the induction and/or effector phase of the immune response. To address this question, female SJL mice were treated with either E2- or E3-containing pellets at the indicated doses 1 wk before immunization with PLP 139-151. Splenocytes were recovered 10 days after immunization and stimulated in vitro with PLP139-151 for 72 h before transfer of 30 million T cell blasts into naive female hosts. The adoptive transfer of placebo-treated splenocytes resulted in the rapid development of severe paralysis, whereas the onset of disease was delayed and significantly less severe in mice receiving splenocytes from estrogen-treated mice (Table V). No differences in protection were observed between equivalent doses of E2 and E3, but splenocytes from high-dose E3-treated mice were the least encephalitogenic. These data support the hypothesis that estrogen has profound effects on the development of the nascent immune response and is consistent with the hypothesis that sex hormones in the lymphoid microenvironment during the initial contact with Ag play a critical role in determining the nature of the response.

Low-dose estrogen treatment at the onset of EAE fails to reduce clinical paralysis

The ability of low-dose estrogen therapy to treat established disease was also investigated. Female SJL mice were immunized with PLP139-151 and treated with low-dose E2- and E3-containing pellets at the onset of clinical disease (day 11). Treatment with low-dose E2 failed to alter the severity of established disease (Table V). Clinical score, peak clinical disease, and CDI were calculated by using the two-tailed Student t test.

### Table III. E2 treatment inhibits EAE induced by immunizing B10.PL mice with Ac 1–11

<table>
<thead>
<tr>
<th>E2 (mg/pellet)</th>
<th>Incidence</th>
<th>Onset (days)</th>
<th>Peak</th>
<th>CDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>26/30 (87%)</td>
<td>13.6 ± 0.6</td>
<td>4.6 ± 0.34</td>
<td>39.3 ± 7.3</td>
</tr>
<tr>
<td>(diestrus)</td>
<td>p = 0.125p</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>0.36</td>
<td>15/26 (58%)</td>
<td>26.5 ± 1.3</td>
<td>2.61 ± 0.60</td>
<td>13.5 ± 6.2</td>
</tr>
<tr>
<td>(estrus)</td>
<td>p = 0.030p</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>2.50</td>
<td>0/15 (0 %)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>(pregnancy)</td>
<td>p &lt; 0.0001p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>15.0</td>
<td>2/8 (25 %)</td>
<td>29 ± 1.4</td>
<td>2.0 ± 1.0</td>
<td>1.4 ± 3.8</td>
</tr>
<tr>
<td>(high)</td>
<td>p = 0.002p</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

*a CDI is defined as the mean of the sum of the daily scores.
*b Significant differences in disease incidence between placebo- and estrogen-treated mice were determined by χ² analysis, and significant differences in disease onset, severity at peak of disease, and CDI were determined using the two-tailed Student t test.
VI), and although there was some indication that E3 at higher doses could reduce severity of disease, these results failed to achieve statistical significance. Estrogen treatment at the onset of paralysis in the B10.PL model also failed to significantly alter the disease course (Table VI). These results show that established disease is relatively resistant to regulation by estrogens and suggest that naïve cells are more sensitive to the effects of sex hormones.

Mechanisms governing estrogen-mediated regulation of EAE

Given that estrogen therapy reduced the capacity of myelin-reactive T cells to transfer disease, our attention focused on investigating estrogen-regulated properties of T cells. mAbs specific for adhesion and activation markers were used to assess whether estrogen therapy altered their expression of T cell adhesion or activation markers. DLN cells were recovered from mice during the peak of clinical EAE and incubated with the indicated fluorochrome-conjugated mAbs, and surface expression was measured by FACS. Approximately 50% (42–49%) of the DLN cells were CD4⁺, and no differences between placebo- and estrogen-treated mice were found (data not shown). Approximately 5% of the CD4⁺ T cells in the DLN had an activated phenotype (CD25⁺, CD69⁺, FasL⁺) but, again, no differences between placebo- and estrogen-treated mice were noted (data not shown). In addition, no differences in adhesion molecule expression (CD44, CD62L, CD49d) were observed (data not shown). These data indicate that estrogen therapy had no apparent effect on the phenotype of T lymphocytes in the lymph nodes draining the site of immunization.

Proliferation of DLN T cells from placebo- and estrogen-treated mice was measured to determine whether estrogen therapy altered the ability of these cells to respond to Ag. DLN T cells were removed from representative animals during the peak of clinical EAE and stimulated with Ag in vitro, and proliferation was measured using a standard [3 H]thymidine incorporation assay. A modest decrease in proliferation to PLP 139-151 was consistently observed in DLN cells isolated from estrogen-treated mice (Fig. 5). However, in all cases the reduction in Ag-specific proliferation failed to achieve statistical significance (p > 0.10). No consistent differences in background or mitogen-induced proliferation were observed.

Table IV. E3 treatment inhibits EAE induced by immunizing B10.PL mice with Ac1–11

<table>
<thead>
<tr>
<th>E3 (mg/pellet)</th>
<th>Incidence</th>
<th>Onset (days)</th>
<th>Peak</th>
<th>CDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>3/4 (75%)</td>
<td>9.7 ± 0.43</td>
<td>4.3 ± 1.4</td>
<td>30.5 ± 30.5</td>
</tr>
<tr>
<td>1.5</td>
<td>3/8 (38%)</td>
<td>30.3 ± 0.9</td>
<td>1.38 ± 0.8</td>
<td>1.9 ± 1.7</td>
</tr>
<tr>
<td>(low)</td>
<td>p = 0.551b</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>5</td>
<td>3/8 (38%)</td>
<td>31.3 ± 0.7</td>
<td>0.71 ± 0.4</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>(pregnancy)</td>
<td>p = 0.551b</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

a CDI is defined as the mean of the sum of the daily scores.
b Significant differences in disease incidence between placebo- and estrogen-treated mice were determined by χ² analysis, and significant differences in disease onset, severity at peak of disease, and CDI were determined using the two-tailed Student t test.
observed (data not shown). A similar modest but insignificant reduction in Ag-specific proliferation was also observed in the B10.PL model (data not shown).

Previous studies have demonstrated a shift toward Th2 immunity during pregnancy and high-dose estrogen therapy (20–22). To determine whether low-dose estrogen therapy altered cytokine secretion patterns, DLN cells were prepared from individual mice at the peak of clinical EAE (day 12–15 postimmunization), and cytokine levels were measured 48–72 h after in vitro stimulation with PLP_{139-151}, IFN-γ, IL-12, and TNF-α were used as representative Th1 cytokines, whereas IL-4 and IL-10 were used as representative Th2 cytokines. Even though the secretion of IFN-γ was consistently lower in E2- and E3-treated groups of mice (Fig. 6A), the reduction in IFN-γ levels fell short of being statistically significant (p > 0.10). The decrease in IFN-γ secretion was accompanied by a modest increase in IL-10 (Fig. 6B) and a small decrease in IL-12 (Fig. 6E). Despite the lack of statistical significance, the trend toward higher Th1 and lower Th2 cytokines points toward a subtle shift in the Th1/Th2 balance. The shift can be seen more clearly when the cytokine response of each individual mouse is plotted as a ratio of IFN-γ to IL-10 (Fig. 6C). There was a marked decrease in the frequency of high Th1 responder mice in the E2- and E3-treated groups when compared with placebo animals that approached significance (p = 0.09 for the 5.0 mg E3-treated mice). We failed to detect any informative trends in IL-4 secretion (Fig. 6D), and TNF-α secretion was very often below the limits of detection for the assay (<31.25 pg/ml). Modest changes in cytokine responses induced by low-dose estrogen therapy were also observed in the B10.PL model and were consistent with the data described for the SJL model (data not shown). Finally, the level of cytokines produced by splenocytes was measured in cultures before the adoptive transfer of EAE (organisms harvested 10 days postimmunization). The influence of E2 and E3 on cytokine production in splenocytes was comparable to DLN cells (data not shown).

Steroid hormones have been shown to alter the sensitivity of immunocompetent cells to apoptotic signals (31). Therefore, we sought to determine whether lymphocytes from estrogen-treated mice were more sensitive to apoptosis. Female SJL mice were treated with either E2 (2.5 mg) or E3 (5.0 mg) for 1 wk before immunization with PLP_{139-151}. DLN cells were recovered 10 days later, and an aliquot of cells was stained with anti CD4-FITC, Annexin V-PE, and 7-amino actinomycin D. FACS analysis revealed a low frequency of CD4^+ T cells (5.4 ± 0.16%) with an early apoptotic phenotype (annexin V^+/7AAD^−) in untreated animals and no difference in the frequency between untreated and estrogen-treated groups (p > 0.531). An aliquot of cells was stimulated with PLP_{139-151} in vitro for 72 h and stained as described above. Although there was an increase in early apoptotic CD4^+ T cells in the untreated group (15.3 ± 0.8%), there was no significant difference between untreated and estrogen-treated groups (p > 0.488). Furthermore, no differences in annexin V staining between untreated and estrogen-treated splenocytes were observed (data not shown). These data imply that estrogen does not increase the sensitivity of lymphocytes for apoptotic signals.

An increase in humoral immunity has been reported during pregnancy and during high-dose estrogen therapy (20–22). Consequently, the humoral immune response in low-dose estrogen-treated animals was compared with placebo controls. Serum was collected from individual mice at the peak of clinical disease and PLP_{139-151}-specific Ig levels were measured using a standard ELISA. Although a considerable amount of anti-PLP_{139-151} activity could be measured, no significant differences (p ≥ 0.180) in PLP_{139-151}-specific Ab production were observed between

<table>
<thead>
<tr>
<th>Donor Treatment</th>
<th>Incidence</th>
<th>Mortality</th>
<th>Onset</th>
<th>Peak</th>
<th>CDI^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>8/8</td>
<td>3/8</td>
<td>6.1 ± 0.5</td>
<td>5.5 ± 0.2</td>
<td>91.4 ± 8.6</td>
</tr>
<tr>
<td>0.36 E2</td>
<td>4/7</td>
<td>0/4</td>
<td>11.8 ± 2.5</td>
<td>3.8 ± 1.9</td>
<td>19.9 ± 8.6</td>
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<tr>
<td>p value^d</td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.248</td>
<td>&lt;0.0001</td>
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<td>2.5 E2</td>
<td>4/5</td>
<td>0/5</td>
<td>8.8 ± 0.4</td>
<td>4.0 ± 0.5</td>
<td>24.9 ± 8.8</td>
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<td>p value</td>
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<td>&lt;0.0001</td>
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<tr>
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<td>0/4</td>
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<td>4.0 ± 0.5</td>
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</tr>
<tr>
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<tr>
<td>5.0 E3</td>
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<tr>
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^a Twenty-five day cumulative disease index ± SD.

^d Significant differences between placebo- and estrogen-treated cell transfer as determined by the two-tailed Student t test.

The table summarizes the cumulative results of at least two separate experiments.
placebo- and estrogen-treated groups (data not shown). These data suggest that the modest shift toward Th2 cytokine production in estrogen-treated mice was insufficient to enhance humoral immunity.

Discussion

Immune reactivity is greater in females than in males, which may explain their increased incidence of autoimmune disease (32, 33). This gender dimorphism in immune responsiveness could have several explanations, including the influence of sex chromosomes, sex hormones, or a combination of genetic and hormonal factors.

A number of studies support the hypothesis that the increased female susceptibility to autoimmune disease is largely due to effects mediated by estrogen (34–36). However, it is well known that pregnancy levels of estrogen are immunosuppressive and protect against a number of experimental autoimmune disorders including collagen-induced arthritis (37, 38) and EAE (16, 17). To explain this apparent dichotomy, it has been proposed that the response to estrogen is biphasic, with basal levels of hormone leading to conditions favoring autoimmunity and pregnancy levels of hormone associated with protection from autoimmune disease (23). However, the exceedingly low levels of estrogen required to achieve significant reductions in clinical EAE appear to challenge this hypothesis. Perhaps the continuous release of estrogen as opposed to the cyclical production associated with the physiological production of sex hormones favors protection from EAE. In contrast, it is also possible that basal levels of estrogen are protective. This idea is consistent with recent data demonstrating an increase in susceptibility and severity of EAE in ovariectomized female mice (39) and lends support to the notion that increased female susceptibility to autoimmune disorders can be controlled, at least in part, at the genetic level.

Alterations in the lymphoid microenvironment during priming with Ag are thought to influence the outcome of the subsequent immune response (40, 41). A reduction in the capacity of lymphocytes from estrogen-treated mice to transfer EAE was observed, and is consistent with the hypothesis that estrogen in the lymphoid microenvironment can alter T cell function. Estrogen has been shown to alter the function of various immunocompetent cells including B cells (42, 43), T cells (30), macrophages (44, 45), NK cells (46), endothelial cells (47, 48), and stromal cells (49). Much attention has been focused on the ability of estrogen to regulate T cell cytokine secretion. It is generally accepted that EAE and other models of organ-specific autoimmunity are mediated by Th1 cells secreting proinflammatory cytokines (IFN-γ, TNF-α, LT, IL-2), whereas Th2 cells produce anti-inflammatory cytokines (IL-4, IL-5, IL-10, TGF-β) and act to regulate disease (9, 10). High-dose estrogen therapy has been shown to bias cytokine responses toward Th2 (20). However, the skewing of T cell activation from a Th1 to Th2 phenotype is most likely not the full explanation of how low-dose estrogen protects against EAE. Despite the profound effects of estrogen treatment on the clinical course of active EAE, only a modest shift toward Th2 cytokine production was measured, an effect that is consistent with previous studies by our group (39).

Furthermore, estrogen therapy failed to enhance the sensitivity of lymphocytes to apoptotic stimuli. It is likely that estrogen therapy alters the expression of many genes related to encephalitogenicity, and the discovery of estrogen-regulated genes is a subject of investigation in our laboratory.

The effector phase of the immune response is also sensitive to regulation by estrogen. Treatment of host mice has been shown to reduce the severity of EAE induced by the adoptive transfer of encephalitogenic lymphocytes (20). In this report, a significant reduction in the number of inflammatory and demyelinating lesions was observed in the CNS of low-dose estrogen-treated mice. This is consistent with a role for estrogen in regulating leukocyte homing and tissue damage in the CNS. This concept is supported by the observation that estrogen can regulate the expression of endothelial cell adhesion molecules such as intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin and alter the binding of leukocytes to cultured endothelial cell monolayers (47). Estrogen replacement therapy is currently being considered as a potential treatment for a number of neurodegenerative disorders including Alzheimer’s and Parkinson’s diseases. This is based on the known ability of estrogen to protect against oxidative injury to neural tissue (50). Consequently, low-dose estrogen therapy may serve to regulate leukocyte entry and reduce oxidative injury in the CNS.

Sex hormones can act on the immune system in a variety of ways. A number of studies have demonstrated that estrogens can directly alter immunocompetent cell function (36, 51), a hypothesis that is supported by evidence demonstrating ERs in Ag-responsive cell types (14, 29). In contrast, estrogen may also act indirectly or mediate interactions with immunomodulatory factors. For example, the influence of estrogen upon the hypothalamic-pituitary-adrenal axis (HPA) is well recognized. Females have an enhanced HPA response to endotoxin compared with males, which is reversed after ovariectomy and is enhanced with estrogen treatment (52, 53). The regulation of glucocorticoid production by estrogen is one likely pathway by which estrogen therapy protects mice from EAE. However, female animals have been shown be more sensitive to estrogen-induced regulation of the HPA than males (54). Estrogen therapy was equally effective in protecting both male and female mice from EAE, suggesting that estrogen regulates the immune response directly or through some other pathway distinct from the HPA.

There are a variety of sex hormones in females that have potential immunomodulatory functions. E2, E3, and progesterone
have received the most attention as of late because they are secrete at high levels during pregnancy, a time marked by a significant reduction in the clinical symptoms of MS (7). A recent comparison of E3 and progesterone demonstrated that although E3 was quite effective at reducing the clinical signs of EAE, progesterone failed to have any significant affect (20). Others have claimed that E3 might be a more potent treatment for EAE than E2 (19), but the small number of animals used and the relatively modest difference in the incidence of clinical disease make it difficult to draw any substantive conclusions. In this study a direct comparison between E2 and E3 revealed no significant differences in the ability of these hormones to reduce the incidence or severity of EAE. These observations were made in a large cohort of mice and in two substantially different EAE models. Because E2 has a higher affinity for both ERs (10-fold higher for ER-α and 5-fold higher for ER-β), and a significantly longer serum half-life than E3 (55), it would seem to be a logical choice as a potential therapy for human autoimmune diseases.

The challenge of using sex hormone-based therapy for autoimmune disease is to maximize the effects of this treatment on the immune system and minimize the effects on other systems, including the reproductive and circulatory systems. The results of this study demonstrate that estrogen dramatically reduces severity of EAE at doses significantly lower than those previously established. These results suggest that estrogen may be an effective treatment for autoimmune disorders at doses that minimize the adverse effects of hormone therapy. Moreover, supplemental estrogen in combination with TCR peptide therapy, or other methods of immunotherapy, may very well be complimentary and could potentially be used together as an effective treatment for MS.

References


