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*J Immunol* 2003; 170:1548-1555; doi: 10.4049/jimmunol.170.3.1548

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Oral Feeding with Ethinyl Estradiol Suppresses and Treats Experimental Autoimmune Encephalomyelitis in SJL Mice and Inhibits the Recruitment of Inflammatory Cells into the Central Nervous System

Sandhya Subramanian,* Agata Matejuk,†† Alex Zamora,* Arthur A. Vandenbark,*†† and Halina Offner2*††

There is much interest in the possible ameliorating effects of estrogen on various autoimmune diseases. We previously established the protective effects of 17β-estradiol (E2) on experimental autoimmune encephalomyelitis (EAE). In the current study we investigated the effectiveness of oral treatment with ethinyl estradiol (EE) on EAE and the mechanisms involved. Ethinyl estradiol is a semisynthetic estrogen compound found in birth control pills, and its chemical structure allows this compound to retain activity when given orally. We found that oral EE, like E2, drastically suppressed EAE induced by proteolipid protein 139–151 peptide when given at initiation of EAE. However, unlike E2, EE reduced clinical severity when given after the onset of clinical signs. Treatment with EE significantly decreased the secretion of proinflammatory cytokines (IFN-γ, TNF-α, and IL-6) by activated T cells as well as the expression of a key matrix metalloproteinase, disease-mediating chemokines/receptors, and IgG2a levels, but increased the expression of TGF-β3 in the CNS. The absence of infiltrating lymphocytes together with the suppression of cytokines, matrix metalloproteinase, and chemokines/receptors suggests that EE, like E2, protects mice from EAE by inhibiting the recruitment of T cells and macrophages into the CNS. These results suggest that oral ethinyl estradiol might be a successful candidate as therapy for multiple sclerosis.


Multiple sclerosis (MS) is a chronic, debilitating, inflammatory disease of the CNS characterized by damage to the CNS myelin (1). A gender bias is evident in disease occurrence; the incidence of MS is higher in women than in men (2, 3). MS patients go into remission during pregnancy, and fewer relapses are observed during this period, which is marked by an increase in the production of sex hormones. In the period following delivery, when pregnancy hormones return to baseline levels, the disease exacerbates within 2 wk to 6 mo postpartum (4, 5).

Experimental autoimmune encephalomyelitis (EAE), an animal model for MS, is an inflammatory disease of the CNS that is induced by immunizing with myelin proteins or peptides (6, 7). Activated CD4+ Th1 lymphocytes specific for myelin Ags traverse the blood-brain barrier and enter the CNS, where they attract macrophages and cause inflammatory lesions (8). The influence of sex hormones as seen in MS is also evident in the animal model EAE. Both male and female mice develop severe EAE following active induction with myelin peptides, but male mice are more resistant to relapses than female mice (9). Also, T cell lines from female mice induced severe EAE in both male and female recipients, whereas Ag-specific T lymphocytes derived from male spleens transferred a less severe disease (10, 11).

Pregnancy and hormones associated with pregnancy exert a protective effect on disease course (12–14). Pregnant animals showed less severe disease than nonpregnant mice (14, 15), and treatment at disease onset with estriol inhibited the severity of passively induced EAE (16). Previous data from our laboratory indicate that treatment with low doses of 17β-estradiol (E2) at the time of disease induction suppressed EAE in C57BL/6, SJL, and B10.PL mice, but E2 treatment was not effective when initiated after the onset of clinical signs (17, 18). Controlled release pellets of E2 significantly suppressed the production of inflammatory Th1 cytokines and TNF-α production in the CNS without an apparent shift to the Th2 cytokine pattern (18). Conversely, oral treatment of mice with E2 did not exhibit the protective effects discussed above (H. Offner, unpublished observations).

17α-Ethinyl estradiol (EE) is a semisynthetic estrogen compound that is a component of birth control pills. The ethinyl group in position C17 of the steroid molecule ring prevents it from enzymatic degradation in the liver, which allows this compound to retain activity after oral administration (19). However, structural differences preclude detection of EE using Abs to E2. Earlier studies involving s.c. treatment with EE in rats demonstrated partial suppression of EAE (20, 21). However, no significant differences in blood leukocyte populations were found between treated and untreated animals (20). The purpose of the current study was to test...
the inhibitory activity of EE as a noninjectable, orally active treatment for EAE. In this report we demonstrate for the first time the protective effects on EAE of orally administered EE, when administered at the time of disease induction protected from EAE, allowing expression of only mild disease in recipient SJL mice, and treatment after onset of clinical signs significantly ameliorated EAE. Our results suggest that EE protects mice from developing EAE by down-regulating inflammatory factors and inhibiting the migration of inflammatory cells into the CNS.

Materials and Methods

**Mice**

Female SJL mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6–7 wk of age. The mice were housed at the animal resource facility at Portland Veterans Affairs Medical Center in accordance with institutional guidelines.

**Antigens**

Mouse proteolipid protein (PLP)_{139–151} (HCLGKWGLHPDKF) was synthesized using solid phase techniques and was purified by HPLC at Beckman Institute, Stanford University (Palo Alto, CA).

**Induction of EAE**

SJL mice were inoculated s.c. in the flanks with 0.2 ml of an emulsion containing 150 µg of PLP_{139–151} peptide and an equal volume of CFA containing 150 µg of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). The mice were assessed daily for signs of EAE according to the following scale: 0 = normal; 1 = limp tail 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 mild forelimb weakness or moderate ataxia; 5 = paraplegia with no more than moderate forelimb weakness; and 6 = paraplegia with severe forelimb weakness or severe ataxia or death.

**Estrogen treatment**

For the suppression studies SJL mice were orally fed daily doses of either 0.1 ml of olive oil (control) or 50 µg of EE (catalogue no. E-4876; Sigma-Aldrich, St. Louis, MO). Another group of mice was implanted with a 60-day release pellet containing 2.5 mg of E2 s.c. in the scapular region behind the neck using a 10-gauge trochar as described by the manufacturer (Innovative Research of America, Sarasota, FL). The mice were implanted or fed starting the day of immunization. All in vitro data were generated from suppression experiments. In the treatment experiments mice were fed 0.1 ml of olive oil (control), 50 µg of EE, or 200 µg of EE starting at disease onset. Mice were fed daily for the duration of the experiment.

**Histopathology**

The intact spinal cord was removed from mice at the peak of clinical disease and was fixed in 10% formalin. Spinal cords were dissected after fixation and were embedded in paraffin before sectioning. The sections were stained with Luxol Fast Blue/periodic acid-Schiff/H&E to assess demyelination and inflammatory lesions or with silver nitrate and analyzed by light microscopy. Semi-quantitative analysis of inflammation and demyelination was determined by examining at least 10 sections from each mouse.

**Proliferation assay**

Draining lymph nodes and spleens were harvested from mice at the peak of clinical disease (days 13–16 postimmunization). A single-cell suspension was prepared by homogenizing the tissue through a fine mesh screen. Cells were cultured in a 96-well, flat-bottom tissue culture plate at 4 × 10^6 cells/well in stimulation medium either alone (control) or with test Ags (PLP_{139–151} Peptide) at varying concentrations. Cells were incubated for 3 days at 37°C. After 3 days cells were cultured in medium containing 150 µg/ml of PLP_{139–151} peptide for 24 h, the last 5 h in the presence of brefeldin A. The cells were then washed and stained with anti-mouse CD4 CyChrome and anti-mouse CD11b FITC for 30 min at 4° C, then washed twice with staining medium (1 X PBS, 2% FBS, and 0.02% NaN3), fixed, and permeabilized with Cytofix/Cytopermin solution (BD Pharmingen, San Diego, CA). The cells were then washed twice in perme/wash buffer (BD Pharmingen) and once in staining medium before three-color FACS analysis on a FACSscan instrument (BD Biosciences) using CellQuest software (BD Biosciences). For each experiment, cells were stained with isotype control Abs to establish background staining and to set quadrants before calculating the percentage of positive cells.

**Cytokine determination by cytometric bead array (CBA)**

Draining lymph nodes and spleens were cultured at 4 × 10^6 cells/well in a 24-well, flat-bottom culture plate in stimulation medium with 2 µg/ml PLP_{139–151} peptide for 48 h. Supernatants were then harvested and stored at –70 °C until tested for cytokines. Mononuclear cells from brain were isolated on a Percoll density gradient as previously described (9) and were cultured at 2 × 10^6 cells/well along with 2 × 10^4 irradiated splenocytes (as APC)/well. Cells were stimulated with 2 µg/ml PLP_{139–151} peptide for 48 h. Supernatants were harvested and stored at –70 °C until tested for cytokines.

**IL-10 and IL-6 detection by ELISA**

IL-10 and IL-6 production from cultured spleen supernatants was determined using the murine IL-10 and IL-6 Quantikine kit (R&D Systems, Minneapolis, MN). Briefly, 100 µl of culture supernatant was added to each well and incubated at room temperature for 2 h. The plate was then washed three times with wash buffer, followed by incubation with 100 µl of anti-mouse cytokine conjugate for 2 h. At the end of incubation the plate was washed, and 100 µl of substrate solution was added. The reaction was stopped using stop solution, and OD was measured at 450 nm. Standard curves for each assay were generated using the recombinant standard provided in the kit. The concentration of cytokine in the supernatants was determined by interpolation from the appropriate standard curve.

**IgG1 and IgG2a detection in serum by ELISA**

Serum was collected from control, 50 µg EE-treated, and 2.5 mg EE-treated mice at the peak of clinical disease, and IgG1 and IgG2a Ab levels were measured by ELISA. Ninety-six-well plates were coated with 10 µg/ml PLP_{139–151} peptide in 1 × PBS overnight at 4° C. Plates were washed and then blocked with blocking buffer (1 × PBS, 2% BSA, and 0.05% Tween 20) for 2 h at 37°C. Plates were washed, followed by addition of 100 µl of sample/well in triplicate. Samples were incubated at 37°C for 2 h and then washed. One hundred microliters of 1/5000 diluted anti-IgG1 or IgG2a-biotin was added to the samples and incubated at room temperature for 1 h. Plates were then washed and incubated for 30 min with 100 µl of 1/400 diluted HRP solution. Samples were washed, followed by the addition of 100 µl of 3,3’,5’,5’-tetramethylbenzidine chromogen solution. The plates were allowed to develop for 30 min, and the reaction was stopped by adding 100 µl of stop solution. The OD was then measured at 490 nm.

**Intracellular staining for cytokines**

Single-cell suspensions from spleen were prepared from all three experimental groups and were cultured at 1–2 × 10^6 cells/ml in stimulation medium containing 10 µg/ml PLP_{139–151} peptide. The cells were stimulated for 24 h, the last 5 h in the presence of brefeldin A. The cells were then washed and stained with anti-mouse CD4 CyChrome and anti-mouse CD11b FITC for 30 min at 4° C, then washed twice with staining medium (1 X PBS, 2% FBS, and 0.02% NaN3), fixed, and permeabilized with Cytofix/Cytopermin solution (BD Pharmingen, San Diego, CA). The cells were then washed with PE-labeled anti-cytokine Abs (anti-mouse TNF-α and IFN-γ) for 30 min at 4° C. Cells were washed twice in perm/wash buffer (BD Pharmingen) and once in staining medium before three-color FACS analysis on a FACSscan instrument (BD Biosciences) using CellQuest software (BD Biosciences). For each experiment, cells were stained with isotype control Abs to establish background staining and to set quadrants before calculating the percentage of positive cells.

CNS mononuclear cells were isolated from brain by Percoll gradient centrifugation as previously described (9). The cells were stimulated with 10 µg/ml PLP_{139–151} peptide for 24 h, the last 5 h in the presence of brefeldin A. Cells were then blocked with anti-mouse FcR (CD16/CD32) and stained with anti-mouse CD4 CyChrome and CD11b FITC for 30 min at 4° C. Cells were washed, fixed, and permeabilized in Cytofix/Cytoperm solution (BD Pharmingen) overnight, followed by staining with PE-labeled intracellular cytokines Abs (anti-mouse TNF-α and IFN-γ) and were analyzed by three-color FACS using CellQuest software. For each experiment cells were stained with isotype control Abs to establish background
staining and to set quadrants before calculating the percentage of positive

cells.

RNase protection assay (RPA)

Spinal cords and spleens were harvested from representative mice at the
peak of clinical disease and were frozen at −70°C until further use. Total
RNA was isolated from three groups of mice using the STAT-60 reagent
(Tel-Test, Friendswood, TX); chemokine and cytokine expression was de-
dtermined using the RiboQuant RPA kit (BD Pharmingen) according to the
manufacturer’s instructions. Custom multiprobe sets detected the following
chemokine transcripts: RANTES, macrophage inflammatory protein-α
(MIP-1α), MIP-2, inducing protein-10 (IP-10), and MCP-1. The cytokines
detected were IL-4 and IL-10. CD4 was also detected. The cytokine probe
set detected TNF-β, IL-12p40, TNF-α, IFN-γ, TGF-β1, TGF-β2, and
TGF-β3. The chemokine receptor probe set detected CCR1, CXCR2,
CCR3, CCR4, CCR5, CCR2, CCR7, CCR8, CXCR3, and CCR6. The sam-
ples were normalized to a housekeeping gene, L32 or GAPDH, included in
the template set. RPA analysis was performed on 5 μg of total RNA hy-
bridized with probes labeled with [α-32P]UTP. After digestion of ssRNA,
the RNA pellet was solubilized and resolved on a 5% sequencing gel. The
gels were analyzed using a phosphorimager (Bio-Rad, Hercules, CA), and
the radioactivity of the experimental bands relative to L32 was determined
using Quantity One software (Bio-Rad).

Zymography assay of matrix metalloproteinases (MMPs)

Spleen and brain mononuclear cells were stimulated in vitro with PLP139–151
peptide for 48 h, and supernatants were harvested and frozen at −80°C until
further use. Culture supernatants were diluted 1/1 in 2× zymogram sample
dilution buffer (catalogue no. LC2676; NOVEX, San Diego, CA) accord-
ing to the manufacturer’s directions. Electrophoresis and staining were
performed following the protocol reported by Kleiner et al. (22). Briefly, 15
ml of diluted sample was loaded onto a precast 10% Tris/glycine gel with
0.1% gelatin incorporated as substrate (NOVEX; catalogue no. LC5925).
The gels also included 3.75 ng of purified MMP-9 and MMP-2 as m.w.
standards. Gels were electrophoresed at 125 V for 2 h, then removed from
the cassette and renatured for 30 min in 1× renaturing buffer (NOVEX;
catalogue no. LC2670) at room temperature with gentle shaking. This was
followed by incubation in 1× developing buffer for 30 min at room tem-
perature on a rotary shaker. The gel was then transferred to fresh 1×
developing buffer and incubated at 37°C for 18 h. Gels were stained in
0.5% Coomassie blue R-250 (Bio-Rad; catalogue no. 161-0400) disso-
lved in 30% methanol/10% acetic acid and destained in the same solution
without dye. Gels were scanned with a Microtek ScanMaker E3 (Vienna,
Austria) scanner using IrfanView software. Scanned images were saved as TIF
files using Adobe Photoshop and were quantified in digital light units using
OptiQuant version 03.10 software.

FIGURE 1. Treatment with EE suppresses EAE in SJL mice. A, SJL females immunized with
PLP139–151/CFA were treated with olive oil (control), 50 μg of EE in olive oil, or a 60-day release,
2.5-mg E2 pellet starting on the day of im-
munization. Mice were treated and scored daily as outlined
in Materials and Methods. Note the nearly complete
protection of the group orally treated with 50 μg of
EE. Data are represented as the mean ± SD of two
experiments for each group. *p < 0.05; **p < 0.01 (signif-
icant difference in CDI between control
vs experimental). B, Treatment with EE at the onset
of clinical signs ameliorates EAE. SJL females were
immunized with PLP139–151/CFA. At disease onset
(day 12), mice were treated with olive oil (control),
50 μg of EE, or 200 μg of EE. Mice were monitored
and treated daily. Data presented are representative
of two experiments for each group. a, Did not die of
EAE. *p < 0.05 (significant difference between
control vs experimental).
Statistical analysis

The day of onset, peak clinical disease score, and cumulative disease index (CDI) among the various groups was evaluated using t test; incidence and mortality rates were compared using χ² test. Comparison of RPA values, cytokine values, and cpm were evaluated by t test; the accepted level of significance was p = 0.05. The MMP values between various groups were evaluated using t test; accepted level of significance was p = 0.05.

Results

Oral feeding with EE protects from EAE and significantly ameliorates disease when administered at disease onset

We have previously shown that 60-day release pellets containing subnephropathy levels of 17β-estradiol prevent EAE in C57BL/6, SJL/J, and B10.PL mice (17, 18). In the current study we investigated the effects of oral feeding with EE on EAE. SJL mice were fed daily with either olive oil (control) or 50 μg of EE in olive oil beginning at the time of immunization. Other mice were implanted with a 2.5-mg, 60-day release pellet of E2 as a positive control. The relative concentrations of active estrogen delivered by the two routes could not be compared, because there is no quantitative assay to detect EE. Moreover, sera from EE-treated mice did not contain increased levels of E2 as assessed by RIA (data not shown).

Immunization with 150 μg of PLP139−151 peptide induced severe EAE in control mice. Mice fed EE displayed a significantly delayed disease onset (day 19.5 ± 0.7) compared with control mice (day 13.3 ± 1.6; Fig. 1A). Also, the disease was less severe in the treated group (lower peak score of 0.6 ± 1.4 compared with 3.0 ± 1.2 in control mice). There were significant reductions in both incidence (4 of 14 vs 16 of 16) and cumulative disease index of EAE (27.5 ± 10.7 vs 3.9 ± 9.6) following oral feeding with EE. Mice treated with the E2 implants also had significantly delayed onset and less severe disease, confirming previous reports (17, 18).

To test treatment effects, mice were immunized with 150 μg of PLP139−151 peptide/150 μg of CFA, and at the onset of clinical disease, mice were fed olive oil (control), 50 μg of EE, or 200 μg of EE. Mice treated with both doses of EE at disease onset showed significant improvement in EAE scores (Fig. 1B; CDI, 38.4 ± 8.5 with 50 μg of EE and 32.7 ± 13.1 with 200 μg of EE) compared with control mice (55.1 ± 4.0), especially after resolution of the first disease episode.

Oral feeding with EE at the time of disease induction down-regulates Th1 cytokine production

To study the mechanism by which EE exerted its protective effects, we isolated brain mononuclear cells and splenocytes from control and oral estrogen-treated mice at the peak of clinical disease. Cells were cultured for 48 h in the presence of PLP139−151 peptide, and the supernatants were assayed for cytokine production by CBA.

Lymphocytes isolated from brains of EE- and E2-treated mice showed a remarkable decrease in the production of TNF-α and IFN-γ (Table I) compared with control mice. A slight, but significant, increase in IL-2 was exhibited in brain cells isolated from EE- and E2-treated mice. Interestingly, there was no difference in IL-5 secretion, whereas a significant decrease in IL-4 secretion was detected in both EE- and E2-treated mice (Table I). Decreased IL-6 and MCP-1 production was detected following EE as well as E2 treatment. No difference in IL-10 levels was detected between the control and EE-treated groups (Table I). Intracellular staining of brain cells confirmed the above results. There was a marked decrease in the percentage of CD4+ TNF-α+ and CD4+ IFN-γ+ T cells in the brains of mice treated with 50 μg of EE as well as the 2.5-mg E2 pellet (Fig. 2).

Splenocytes displayed a different pattern of cytokine secretion than brain. Although there were nominal changes in TNF-α and IL-2 secretion, the changes observed were not significant (Table II). On the other hand, IFN-γ production was strongly enhanced in splenic mononuclear cells in EE- and E2-treated mice compared with control mice (Table II). There was no change in IL-5 production, and there was a significant decrease in IL-4 production (data not shown). However, both EE and E2 induced a >2-fold decrease in IL-6 production. There was also a significant decrease in MCP-1 levels (Table II).

Table I. Oral feeding with EE suppresses production of Ag-specific proinflammatory cytokines in the brain

<table>
<thead>
<tr>
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<th>Control</th>
<th>50 μg of EE</th>
<th>2.5 mg of E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>1306.9 ± 40.7</td>
<td>370.9 ± 8.5b</td>
<td>409.1 ± 21.9b</td>
</tr>
<tr>
<td>IL-2</td>
<td>55.9 ± 2.8</td>
<td>67.8 ± 0.9a</td>
<td>92.4 ± 4.0a</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5000 ± 0.0</td>
<td>906.3 ± 12.6b</td>
<td>1250.6 ± 61.6b</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>8.9 ± 1.3</td>
<td>5.3 ± 0.7</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>IL-4</td>
<td>83.6 ± 3.1</td>
<td>9.0 ± 1.1b</td>
<td>6.7 ± 0.1b</td>
</tr>
<tr>
<td>IL-6</td>
<td>708.3 ± 14.4</td>
<td>86.9 ± 0.6</td>
<td>107.4 ± 4.5b</td>
</tr>
<tr>
<td>IL-10</td>
<td>31.3 ± 6.9</td>
<td>23.9 ± 9.7</td>
<td>16.6 ± 1.8</td>
</tr>
<tr>
<td>MCP-1</td>
<td>4183.0 ± 29.6</td>
<td>1972.8 ± 84.1b</td>
<td>986.1 ± 4.5b</td>
</tr>
</tbody>
</table>

* Cytokine values are expressed in picograms per milliliter. Data shown are representative of two experiments.

a Significant differences between control and experimental, p < 0.05.

b Maximum level of detection using CBA.

Table II. Cytokine production in spleen following EE treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>50 μg of EE</th>
<th>2.5 mg of E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>155.2 ± 5.9</td>
<td>373.3 ± 168.1</td>
<td>281.4 ± 50.1</td>
</tr>
<tr>
<td>IL-2</td>
<td>108.9 ± 9.3</td>
<td>71.5 ± 30.2</td>
<td>49.9 ± 3.6</td>
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<tr>
<td>IFN-γ</td>
<td>395.8 ± 15</td>
<td>3979.6 ± 1178.6b</td>
<td>3430 ± 1812.9b</td>
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<tr>
<td>IL-6</td>
<td>160.4 ± 13.2</td>
<td>60.8 ± 6.3b</td>
<td>33.7 ± 3.2b</td>
</tr>
<tr>
<td>IL-10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5000 ± 0a</td>
<td>2247.1 ± 109.3b</td>
<td>1027.3 ± 31.3b</td>
</tr>
</tbody>
</table>

* Cytokine values are expressed in picograms per milliliter. Data presented are representative of two experiments. ND, not detected.

a Significant differences between control and experimental, p < 0.05.
Ab production in response to treatment with oral estrogen

At the peak of clinical disease (days 14–16), serum was collected from control, 50 μg EE-treated, and 2.5 mg E2-treated mice. The serum was then tested for IgG1 and IgG2a Ab production. A significant decrease in IgG2a production, generally reflective of Th1 activity, was detected following treatment with 50 μg of EE (Fig. 3). E2 treatment also decreased IgG2a production, although not significantly. There were no detectable differences in IgG1 levels among the three groups. These results confirm the cytokine pattern detected in brain, where there was a decrease in the proinflammatory Th1 cytokines.

Oral feeding with EE does not alter the ability of encephalitogenic T cells to respond to Ag, but affects their capability to migrate into the CNS

To determine whether EE could alter the ability of T cells to recognize and proliferate to the immunizing Ag, draining lymph node cells and splenocytes from control and treated mice were isolated and stimulated with PLP139–151 for 72 h. The results clearly indicate that EE had no inhibitory effect on the ability of PLP139–151-specific T cells to proliferate in response to Ag (data not shown).

Culture supernatants from spleen and brain T lymphocytes were assayed for MMP (MMP-9 and MMP-2) expression. The expression of MMP-9 was markedly inhibited in spleen and brain of EE-treated mice (Fig. 4). E2-treated mice displayed suppressed expression of MMP9 in brain, but not spleen. MMP2 levels were not significantly altered in brain of either treatment group (Fig. 4B). No MMP2 was detected in spleen.

EE suppresses cytokine, chemokine, and chemokine receptor mRNA expression in CNS

Oral estrogen treatment was further evaluated for its effects on the expression of chemokines/receptors and cytokines. As shown in Fig. 5A, 50 μg of EE induced a significant decrease in the expression of RANTES and MIP-1α mRNA. The expression of MIP-2, IP-10, and MCP-1 was also suppressed, although not significantly. Treatment with EE dramatically suppressed the expression of chemokine receptors CCR2, CCR3, CCR4, CCR5, and CCR7 (Fig. 5B) and the cytokine TGF-β1 (Fig. 6A), but significantly enhanced the expression of TGF-β3 (Fig. 6B). Although there was a notable decrease in IFN-γ, TNF-α, IL-12, and CD4 mRNA expression, statistical significance was not achieved (Fig. 6A). Treatment with E2 pellets caused an almost identical suppression of chemokines receptors and cytokines in CNS, as previously described (50).

Treatment with EE prevents inflammation and demyelination of the spinal cord

Histopathologic analysis revealed significant reduction in inflammation and demyelinating lesions in the spinal cord of mice treated with 50 μg of EE compared with control mice that had severe inflammation and demyelination (Fig. 7). There was a notable decrease in cell yield from brains of EE-treated mice (~1.6 × 10⁵ compared with 6 × 10⁵ cells/brain from control mice), suggesting that EE treatment prevented the entry of inflammatory cells into the CNS. Cords from mice treated with the E2 pellet also indicated no inflammation or demyelination, confirming previous reports (18).

Discussion

Previous studies from our laboratory and others have established the protective effects of estrogen therapy on EAE when administered during disease induction (13, 16–18). In this study we demonstrate that EE, an orally active estrogen compound found in oral contraceptives, protects against the development of EAE when administered at the time of disease induction, similar to E2. This oral suppression protocol was even more effective than daily s.c. injections of EE given to Lewis rats during disease induction, as reported by Trooster et al. (20). A previous study by Kim et al. (16) demonstrated that estriol, given as a pellet after the onset of clinical signs, could reduce the severity of EAE in mice. The major new finding presented here is that oral EE can also ameliorate the course of EAE when administered after the onset of clinical signs. In contrast, treatment with E2 pellets after disease onset did not alter the clinical course of EAE (H. Offner, unpublished observations). The difference observed between orally administered EE
and s.c. administered E2 may be due to the enhanced stability of EE, which protects it from degradation in the liver. Future studies are in progress to determine whether the therapeutic effects of EE are mediated through E2α or E2β receptors in knockout mice.

The cells responsible for the pathogenicity of EAE have been characterized as Th1 cells that secrete IFN-γ, IL-2, and TNF-α (23). The mechanism involved in the protection conferred by EE appeared to be due to a decrease in Th1 cytokine production, rather than a Th1 to Th2 shift in cytokine secretion. In fact, we were unable to detect any evidence of enhanced secretion of IL-4, IL-5, or IL-10 following treatment of actively induced EAE with EE. Similarly, E2 treatment protected mice from developing EAE by reducing Th1 cytokines without inducing Th2 cytokines (18).

These data indicate that the EE-mediated suppression of EAE probably does not involve Th2 cytokines such as IL-10, as was reported for estriol treatment of passive EAE that did not involve

**FIGURE 5.** EE reduces the expression of chemokines (A) and chemokine receptors (B). Total RNA was purified from spinal cords harvested at the peak of clinical disease using STAT-60 reagent. RPA was then performed using chemokine and chemokine receptor-specific riboprobes labeled with 32P. The protected fragments were resolved on a sequencing gel. Band intensity was quantified using a phosphorimager as described in Materials and Methods. Significant differences between control and experimental groups were determined using Student’s t test (*, p < 0.05).

**FIGURE 6.** Oral feeding with EE suppressed cytokine mRNA expression in the CNS. Total RNA was purified from spinal cords harvested at the peak of clinical disease using STAT-60 reagent. RPA was then performed using cytokine-specific riboprobes labeled with 32P. The protected fragments were resolved on a sequencing gel. Band intensity was quantified using a phosphorimager as described in Materials and Methods. Significant differences between control and experimental groups were determined using Student’s t test (*, p < 0.05).
be a key factor in inducing protection from EAE. Our data and indicate that suppressing the expression of IL-6 may
– development of EAE, and selective inhibition of IL-6 production de-

finitive cytokine (24) and has been reported to play a signi-

estingly, EE and E2 treatment induced a remarkable suppression of
proin-
flammatory cytokines by cells derived from the brain. Inter-

\[ \text{IFN-}\gamma \] and TNF-\[ \alpha \] are secreted by cells in the CNS and a concomitant decrease in the production of these
proinflammatory cytokines by cells derived from the brain. Inter-

ingestingly, EE and E2 treatment induced a remarkable suppression of
IL-6 production in both brain and spleen. IL-6 is a pleiotropic mul-
tifunctional cytokine (24) and has been reported to play a significant
role in the induction of autoimmune diseases such as rheumatoid ar-
thritis and EAE (25, 26). Mice deficient in IL-6 expression resist the
development of EAE, and selective inhibition of IL-6 production de-
layed the onset of EAE in rats (27–29). These reports are consistent
with our data and indicate that suppressing the expression of IL-6 may
be a key factor in inducing protection from EAE.

The SJL/J mice protected with EE or E2 exhibited an increased production of IFN-\( \gamma \) in the spleen at the peak of disease. This pattern was different from our previous observation in C57BL/6 mice showing that E2 treatment decreased IFN-\( \gamma \)-secreting cells in the spleen. These differences might be attributed to strain differences or temporal changes in cytokine expression that reflect compartmentalization of responses in brain vs spleen. IFN-\( \gamma \) has been reported to have regulatory effects in EAE and MS. Studies with IFN-\( \gamma \) knockout mice indicate that these mice are susceptible to EAE (18, 30). Furthermore, administration of IFN-\( \gamma \) protects against demyelination and EAE (31, 32). Thus, activation, differentiation, and homing of T cells to the target tissue might be af-
fected by the differential systemic expression of cytokines (33).

In this report we demonstrate for the first time a modulatory effect of EE on TGF-\( \beta \) expression in EAE. EE treatment had reciprocal effects on TGF-\( \beta \) subtype levels, with a very potent in-
crease in TGF-\( \beta 3 \) and a significant decrease in TGF-\( \beta 1 \) mRNA expression. Interestingly, TGF-\( \beta \) has been shown to exert protective ef-
cfects on the development of EAE (35, 36). TGF-\( \beta 3 \) inhibits the migration and homing of lymphocytes into the CNS (37) and also inhibits the production of Mycobacterium bovis-induced secretion of TNF-\( \alpha \) (38). Such changes might explain the decrease in TNF-\( \alpha \) production that is displayed in the brains of EE- and E2-treated mice. Whether the expression of TGF-\( \beta 1 \) and TGF-\( \beta 3 \) has any relevance to treatment with EE requires further investigation.

Histopathologic analysis of spinal cords indicated a lack of in-
filtrating cells following oral treatment with EE. To address the
question of whether EE exerted an effect on the ability of enceph-

alitogenic T cells to traverse the blood-brain barrier, we studied the
expression of MMPs on brain cells derived from EE-treated mice. MMPs are considered to be physiological mediators of cell migration through various barriers (39). T cells are known to produce MMP-9, also known as gelatinase B. It has been shown that MMP-9 facilitates the migration of T cells across the blood-brain barrier (40). The expression of MMPs is regulated during the course of EAE, with heightened expression at the peak of disease and a return to baseline levels in the recovery phase (41, 42). EE treatment inhibited the expression of MMP-9 in the brain, indicat-
ing a possible mechanism by which the modified estrogen com-

pound protects against EAE.

Elevated levels of the chemokines, RANTES, IP-10, MIP-1\( \alpha \), MIP-1\( \beta \), and MCP-1, and their associated receptors (CCR2 and

CCR5) have been detected in the CNS of animals with EAE (43–
48), produced primarily by infiltrating T cells, macrophages, mi-
croglia, and astrocytes (45). These chemokines and their receptors
semble to be critical in the induction of EAE, since their absence
abrogates disease (44, 46, 47). Furthermore, DNA vaccines against
MIP-1\( \alpha \) and MCP-1 protected against EAE (49). Notably, chemokine and chemokine receptor levels were substantially decreased following low dose estrogen therapy (50). We noted similar effects following oral feeding with EE, suggesting that these estrogen compounds may act similarly by suppressing the production of chemokine and chemokine receptors, thereby preventing the mi-
gration of cells into the CNS. The absence of infiltrating lympho-

cytes in the CNS also supports this hypothesis.

Taken together, our data strongly suggest that EE alters the course of actively induced EAE by down-regulating proinflammatory cyto-
kines and inhibiting the recruitment of pathogenic T cells into the
CNS by modulating the expression of key transmembrane transport-
ers, including disease-associated MMPs and chemokine receptors. This,
in turn, may impede the activation and migration of APCs to the CNS.
microenvironment, thereby preventing inflammation and demyelination of the brain tissue. It should be noted that several studies following cohorts of women taking oral contraceptives did not affect susceptibility for developing MS (51). However, these findings do not preclude its use as a therapeutic for MS.

Acknowledgments

We thank Eva Niesaus for assistance in preparing the manuscript.

References