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*J Immunol* 2009; 183:3672-3681; Prepublished online 26 August 2009; doi: 10.4049/jimmunol.0901351

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Estrogen Controls Vitamin D₃-Mediated Resistance to Experimental Autoimmune Encephalomyelitis by Controlling Vitamin D₃ Metabolism and Receptor Expression

Faye E. Nashold,* Karen M. Spach,† Justin A. Spanier,* and Colleen E. Hayes²*

Multiple sclerosis (MS) is an autoimmune, neurodegenerative disease with a rapidly increasing female gender bias. MS prevalence decreases with increasing sunlight exposure, supporting our hypothesis that the sunlight-dependent hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is a natural inhibitor of autoimmune T cell responses in MS. We found that vitamin D₃ inhibited experimental autoimmune encephalomyelitis (EAE) in intact female mice, but not in ovariectomized females or males. To learn whether 17β-estradiol (E₂) is essential for vitamin D₃-mediated protection, ovariectomized female mice were given E₂ or placebo and evaluated for vitamin D₃-mediated EAE resistance. Diestrus-level E₂ implants alone provided no benefit, but they restored vitamin D₃-mediated EAE resistance in the ovariectomized females. Synergy between E₂ and vitamin D₃ occurred through vitamin D₃-mediated enhancement of E₂ synthesis, as well as E₂-mediated enhancement of vitamin D receptor expression in the inflamed CNS. In males, E₂ implants did not enable vitamin D₃ to inhibit EAE. The finding that vitamin D₃-mediated protection in EAE is female-specific and E₂-dependent suggests that declining vitamin D₃ supplies due to sun avoidance might be contributing to the rapidly increasing female gender bias in MS. Moreover, declining E₂ synthesis and vitamin D₃-mediated protection with increasing age might be contributing to MS disease progression in older women. The Journal of Immunology, 2009, 183: 3672–3681.

The vitamin D endocrine system probably mediates the protective effects of sunlight exposure in MS (9). UV light is required for an early step in the biosynthesis of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), a secosteroid hormone (10). The unique dependence of 1,25-(OH)₂D₃ synthesis on UV light, and the discovery of vitamin D receptors (VDR) in activated T lymphocytes (11, 12), led us to propose that sunlight’s protective effects in MS might reflect the need for 1,25-(OH)₂D₃ to control the T lymphocyte-mediated autoimmune responses that are pathogenic in MS (9, 13).

Since we first proposed this hypothesis, a diverse and compelling body of supporting evidence has emerged from human studies (14). For example, high levels of serum 25-hydroxyvitamin D₃ (25-(OH)D₃), which is biologically inactive, correlated with low MS risk, fewer relapses, and significantly less disability (15–21). Conversely, low 25-(OH)D₃ levels correlated with high MS risk, frequent relapses, and significantly higher disability. In an ongoing vitamin D₃ intervention study, investigators noted a trend toward fewer relapses and stable or improved disability in the supplemented patients compared with the unsupplemented MS patients (22). There is a 3–4 mo lag between the seasonal decreases in UV light exposure and circulating 25-(OH)D₃ levels and the increases in MS relapses (17, 19, 20). This lag suggests that seasonal fluctuations in UV light exposure could contribute to the relapsing-remitting MS disease phenotype. Importantly, low circulating levels of biologically active 1,25-(OH)₂D₃ correlated with a progressive disease course (21). Consistent with the possibility that reduced 1,25-(OH)₂D₃ synthesis might be causal in MS progression, a recent report described three patients with vitamin D-dependent rickets type I who all developed MS (23). Vitamin D-dependent rickets type I is a very rare hereditary disease caused by mutation of the Cyp27b1 gene encoding the enzyme that converts inactive 25-(OH)D₃ into 1,25-(OH)₂D₃. The probability of three coincident vitamin D-dependent rickets type I and MS cases by chance alone is ~3.4 × 10⁻⁹, arguing strongly for a cause-effect relationship between Cyp27b1 mutation, reduced 1,25-(OH)₂D₃...
synthesis, and MS disease. Finally, a clinical trial found that MS patients had 80% fewer relapses and no disability progression while they were receiving 1,25-(OH)\textsubscript{2}D\textsubscript{3} treatment, compared with their relapse frequency and disability progression rates before and after treatment (24). Taken together, these human studies strongly support our hypothesis that low 25-(OH)D\textsubscript{3} and 1,25-(OH)\textsubscript{2}D\textsubscript{3} levels increase MS disease risk, severity, and progression (9).

There is a rapidly increasing female gender bias in MS that is not understood (25–30). This female gender bias involves sex hormones, because it becomes apparent after sexual maturity (31). It is not clear why the female-to-male sex ratio rises with increasing estrogen synthesis in sexually mature females, because estrogen is protective in both human MS and rodent experimental autoimmune encephalomyelitis (EAE) (32–36). In our EAE studies, we found a link between female sex hormones and vitamin D. High-level vitamin D\textsubscript{3} supplementation inhibited EAE in intact adult female mice, but not in ovariectomized (OVX) females or males (37). Thus, vitamin D\textsubscript{3} provided a female-specific and sex hormone-dependent protective effect in EAE. Interestingly, high serum 25-(OH)D\textsubscript{3} levels correlated with a reduced MS risk in women but not in men (38). If vitamin D\textsubscript{3} were to provide a female-specific and sex hormone-dependent protective effect in MS as in EAE, then this protective effect would become evident at sexual maturity, and after puberty, vitamin D\textsubscript{3} insufficiency would increase MS risk in a female-specific manner.

Here, we investigated the female-specific and sex hormone-dependent protective effect of vitamin D\textsubscript{3} in EAE in more detail. To test the hypothesis that 17β-estradiol (E\textsubscript{2}) might regulate vitamin D\textsubscript{3} metabolism and/or VDR expression in the CNS, OVX female mice were implanted with E\textsubscript{2} or placebo pellets and evaluated for vitamin D\textsubscript{3}-mediated EAE resistance, vitamin D\textsubscript{3} metabolism, and VDR expression in the CNS. Our data show for the first time that E\textsubscript{2} enables vitamin D\textsubscript{3} to decrease EAE risk and severity in female mice through enhancement of VDR transcription and function in the CNS. We propose a model for the functional synergy between the vitamin D and estrogen endocrine systems, and we discuss our model in the context of the relapsing-remitting phenotype, the female gender bias, and vitamin D\textsubscript{3}-based strategies to decrease MS risk and disease severity.

Materials and Methods

Mice
The B10.PL-H\textsuperscript{2}H\textsuperscript{2}-H\textsuperscript{2}T18\textsuperscript{a}/73NS/SN/J (hereafter B10.PL) and C57BL/6 (hereafter B6) mice were obtained from The Jackson Laboratory. Some B10.PL mice were bred in the pathogen-free mouse colony of the Department of Biochemistry from The Jackson Laboratory-derived breeding pairs. Mice were housed at 25°C with a 12 h light-dark cycle and 40–60% humidity. The drinking water was provided ad libitum. Before experiments, the mice were fed commercial mouse chow containing 0.33 μg/day of vitamin D\textsubscript{3} and 1% calcium (Lab Diet no. 5008; PMI Nutrition International). The Institutional Animal Care and Use Committee approved the experimental protocols. All animal experimentation was conducted in accordance with accepted standards of humane animal care.

Experimental diets
The synthetic diet was formulated to contain all essential nutrients except vitamin D\textsubscript{3}, exactly as we described (37). The vitamin D\textsubscript{3} (cholecalciferol; Acros Organics) was dissolved in absolute ethanol (1 mg/ml) and stored in the cold. It was added to the synthetic diet in an amount calculated to provide 0 (−D diet) or 1 μg/day (+D diet) of vitamin D\textsubscript{3} based on a measured daily diet consumption of 4.0 g dry weight of diet per mouse (37). Groups of mice (sex-matched; age 6–8 wk) were fed the −D or +D diet continuously beginning on the first day of each study. Fresh synthetic diet was prepared weekly, stored at 4°C, and provided to the mice three times per week.

Ovariectomy and estradiol therapy
OVX or SHAM surgery was performed 1 wk after synthetic diet feeding began. During the surgery, a controlled release pellet containing E\textsubscript{2} (3 mg; 0.1 mg of E\textsubscript{2}) or a placebo was implanted dorsally s.c. using a 12-gauge trochar, as described by the manufacturer (Innovative Research of America).

EAE induction and evaluation
EAE was induced 3 wk after synthetic diet feeding began. The myelin basic protein (MBP), isolated from guinea pig spinal cords and dissolved in 0.1 M acetic acid (4 mg/ml), was emulsified with an equal volume of CFA containing heat-killed Mycobacterium tuberculosis H37 Ra (4 mg/ml). Each anesthetized B10.PL mouse was injected s.c. with 0.2 ml of emulsion (600 μg of MBP) in three sites on the hind flanks. Each mouse was injected i.p. with 200 ng of pertussis toxin (List Biological Laboratories) in 0.1 ml of sterile PBS solution on the day of MBP immunization and again 2 days later. EAE was induced in B6 mice using the same protocol, except that a peptide from mouse myelin oligodendrocyte protein (MOG\textsubscript{35–55}, sequence MEVGWYRSPFSRVVHLRPRNGK) replaced MBP, and the emulsion injection volume was 0.1 ml (200 μg of MOG\textsubscript{35–55} peptide).

Analysis of E\textsubscript{2}, 25-(OH)D\textsubscript{3}, and 1,25-(OH)\textsubscript{2}D\textsubscript{3}
The serum E\textsubscript{2} was assayed using an enzyme immunoassay kit (DSL 10-4300; Diagnostic Systems Laboratories). Enzyme immunoassay kits were also used to quantify the serum 25-(OH)D\textsubscript{3} (Alpco Diagnostics) and 1,25-(OH)\textsubscript{2}D\textsubscript{3} (ImmunoDiagnostics). The spinal cords were extracted and the extracts assayed in duplicate for 1,25-(OH)\textsubscript{2}D\textsubscript{3} as we described previously (37). A spike-recovery control was performed with each spinal cord extraction, the percentage recovery was calculated, and a recovery correction factor was applied to the experimental data.

Spinal cord transcript analysis
The GAPDH, IFN-γ, VDR, Cyp27b1, and Cyp24a1 transcript abundance was measured in the spinal cord by real-time PCR as described (37). The amplification and detection were accomplished with a 7500 Fast real-time PCR system (Applied Biosystems) and SYBR Advantage quantitative PCR premix (Clontech). Published primers for the GAPDH, IFN-γ, and Cyp27b1 were used (42, 43). The VDR primers were forward 5′-GCA ACA GCA CAT TAT CCC CAT-3′ and reverse 5′-TAC GTC TGC ACG AAT TGG AGG-3′. The Cyp24a1 primers were forward 5′-ACC CCC AAG GTC CGT GAC ATC-3′ and reverse 5′-CCA GTT GGT GGG TCC AGG TAA GG-3′. Primers were purchased from Integrated DNA Technologies or Invitrogen.

To generate a standard curve, CDNA representing each specific amplification was gel purified and quantified by absorbance at 260 nm. Each real-time PCR included reactions with serially diluted standard cDNA. The standard cDNA copy number, calculated from the absorbance and the dilution, was plotted vs the threshold cycle (C\textsubscript{T}). The experiment group copy number in each unknown sample was determined from C\textsubscript{T} by reference to the appropriate standard curve. The data were calculated as transcript abundance relative to GAPDH as the internal standard.

Data analysis
Individual mice were analyzed and the means and SD or SEM were calculated for each group of mice. The group sizes are given in the table and figure legends. The significance of differences between the group means was determined using the Mann-Whitney U test (n ≤ 16). Student’s t test (n > 16), or χ\textsuperscript{2} test (binomial data); p < 0.05 was considered significant.
Results

Estrus-level $E_2$ does not alter EAE disease

The hypothesis that $E_2$ controls vitamin $D_3$-mediated EAE resistance through regulation of vitamin $D_3$ metabolism and/or VDR expression in the CNS predicts that $E_2$ repletion would restore vitamin $D_3$-mediated EAE resistance in O VX females. Therefore, we first determined an $E_2$ repletion level that would not inhibit EAE in OVX mice independently of supplementary vitamin $D_3$. Implanting 0.36 mg of $E_2$ timed-release pellets into OVX B10.PL females inhibited EAE independently of vitamin $D_3$ (44), so we repeated this study with 0, 0.1, or 0.36 mg of $E_2$ implants. Two weeks after OVX and pellet implantation, the mice were immunized with MBP and monitored daily for clinical EAE signs. Uterine tissue was collected and weighed at the end of the study as an indicator of total $E_2$ exposure (Fig. 1A). The SHAM control mice had uterine weights of 0.11 ± 0.05 mg. The OVX mice with placebo pellets had lower uterine weights than did SHAM controls (Fig. 1A), confirming $E_2$ depletion. The OVX mice with 0.1 mg of $E_2$ pellets had uterine weights equal to the SHAM controls, indicating normal $E_2$ levels. Finally, the OVX mice with 0.36 mg of $E_2$ pellets had higher uterine weights than did SHAM controls, indicating high-level $E_2$ repletion (44). Because high-level $E_2$ repletion decreased the EAE incidence, peak severity, and cumulative disability independently of supplementary vitamin $D_3$ (Fig. 1, B and C), but low-level $E_2$ repletion alone had no effect, we used low-level $E_2$ repletion in all subsequent experiments.

Vitamin $D_3$ supplementation increased serum $E_2$ in OVX mice

The next experiments investigated possible interactions between vitamin $D_3$ and low-level $E_2$ repletion in OVX mice. Female B10.PL mice were fed diets with (1 $\mu$g/day; +$D_3$) or without (0 $\mu$g/day; −$D_3$) vitamin $D_3$. This +$D_3$ diet provided 3-fold the amount of vitamin $D_3$ in standard laboratory mouse chow. After 1 wk of −$D_3$ or +$D_3$ diet feeding, OVX or SHAM surgery was performed and 0 or 0.1 mg of $E_2$ pellets was implanted. MBP immunizations were performed 2 wk after surgery. The serum $E_2$ levels were quantified 7, 24, and 48 days after surgery, and uterine weights were measured 48 days after surgery (Fig. 2A). In the OVX/placebo mice, the $E_2$ level declined significantly relative to the SHAM controls 24 days after surgery ($p < 0.005$), but rose by 48 days after surgery due to $E_2$ synthesis by the adrenal glands (45). In the OVX/$E_2$ mice, the $E_2$ level was elevated 7 days after surgery ($p < 0.005$), but matched the SHAM controls 24 and 48 days after surgery. Unexpectedly, when the $E_2$ data were analyzed by dietary vitamin $D_3$ group, there was significantly more $E_2$ in the +$D_3$ OVX/placebo serum samples than in the −$D_3$ OVX/placebo samples ($p < 0.03$) (Fig. 2B). This trend was also evident in the SHAM and OVX/$E_2$ mice, but it did not reach significance due to the variability in serum $E_2$ measurements. The uterine weights confirmed that the OVX mice had lower total $E_2$ exposure than did the SHAM controls, whereas the 0.1 mg of pellet provided a total $E_2$ exposure equal to the SHAM controls (Fig. 2C).

The serum 25-(OH)$D_3$ levels were quantified at the end of the study. The groups ingesting the −$D_3$ diet had significantly less serum 25-(OH)$D_3$ than did the groups ingesting the +$D_3$ diet ($p < 0.01$; Table I). The groups did not differ significantly with respect to terminal weights or serum calcium levels. These data are consistent with our published data (37), confirming that this level of vitamin $D_3$ supplementation does not cause hypercalcemia.

Having confirmed that the serum $E_2$ and 25-(OH)$D_3$ levels differed between the respective pellet and dietary groups as intended, we next analyzed the EAE data. Consistent with our published report, the vitamin $D_3$ significantly reduced the incidence 28% ($p < 0.05$), delayed disease onset by 10 days ($p < 0.05$), decreased the peak severity ~50% ($p < 0.05$), and diminished the cumulative disease severity ~70% ($p < 0.05$) in the SHAM females, but provided no benefits whatsoever in the OVX/placebo mice (Fig. 3 and Table II) (37). Extending our published results, we found that diestrus-level $E_2$ repletion restored all of the protective effects of vitamin $D_3$ with respect to EAE incidence, onset, peak severity,
and cumulative disease, although it had no independent effect on EAE disease.

In B10.PL mice immunized with MBP, EAE follows a relapsing-remitting disease course (46), whereas in B6 mice immunized with MOG35–55 peptide, EAE follows a chronic progressive disease course (47). To learn whether E2 and vitamin D3 would cooperate to inhibit chronic progressive EAE as they did to inhibit relapsing-remitting EAE, we repeated the OVX experiments in B6 mice. Female B6 mice were fed −D or −D diets, OVX or SHAM surgery was performed, and 0 or 0.1 mg of E2 pellets was implanted in the OVX mice. The MOG35–55 immunizations were performed 2 wk after surgery. In the SHAM B6 mice, the vitamin D3 reduced the cumulative disease severity by 43% (p < 0.05) compared with the −D SHAM group, and there were trends toward lower incidence and decreased peak severity that did not reach significance (Table II). However, the vitamin D3 provided no benefits whatsoever in the OVX/placebo B6 mice. Comparing the OVX B6 mice with 0.1 mg of E2 implants to the OVX B6 mice with placebo implants, we saw a delay in disease onset and a 36% reduction in the cumulative disease (p < 0.02) that were attributable to the E2 implants independently of dietary vitamin D3. However, comparing the −D OVX/E2 mice to the −D OVX/placebo mice, we found that the −D diet further reduced the cumulative disease by 37% (p < 0.001), and there was a trend toward lower peak severity. The animals ingesting the −D diet had significantly higher spinal cord 1,25-(OH)2D3 levels than did the animals ingesting the −D diet (p < 0.005; Table III), although the serum 1,25-(OH)2D3 levels were not different between the groups (data not shown). These data confirm our report that 1,25-(OH)2D3 synthesis occurred in the inflamed spinal cord, independently of 1,25-(OH)2D3 synthesis in the kidney (37). In summary, E2 repletion provided a benefit that was independent of dietary vitamin D3 in B6 mice, and additionally, E2 repletion restored the ability of dietary vitamin D3 to inhibit EAE disease.

E2 supplementation did not enable vitamin D3 to inhibit EAE in intact male mice

In our previous study, vitamin D3 failed to inhibit MBP-induced EAE in the intact and castrated male B10.PL mice, indicating that male sex hormones did not impede vitamin D3-mediated EAE resistance (37). Here we examined whether E2 implants would enable vitamin D3-mediated EAE resistance in intact male mice. Male B6 mice were fed diets with 0, 1, or 2 μg/day vitamin D3, implanted with MOG35–55 peptide to model the progressive form

<table>
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<th>Serum Caa</th>
<th>Vitamin D3 (μg/day)</th>
<th>E2 Implant (mg)</th>
<th>Terminal Weight (g)</th>
<th>Terminal Weight (g)</th>
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<tr>
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<td>8.2 ± 1.7</td>
<td>10 ± 8*</td>
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</table>

Female B10.PL mice were fed diets with 0 or 1 μg/day vitamin D3 continuously beginning 1 wk before surgery. OVX or SHAM surgery was performed and a placebo or E2 pellet (0.1 mg; 60-day release) was implanted s.c. in each OVX mouse. Two weeks after surgery the mice were immunized to induce EAE as described in Materials and Methods.

Serum Ca and 25-(OH)D3 were quantified at the end of the study as described in Materials and Methods. The data shown are the means ± SD (≥8–7 mice/group) from one experiment of four. *, p < 0.01 and **, p < 0.005 for comparisons to the SHAM controls (Mann-Whitney test).

FIGURE 2. Implantation of diestrus level E2 pellets restored serum E2 and uterine weights to normal levels in OVX mice. A, Serum E2 concentration 7, 24, and 48 days after pellet implantation. Female B10.PL mice were fed diets with 0 or 1 μg/day vitamin D3. One week later, OVX or SHAM surgery was performed and a placebo or E2 pellet (0.1 mg; 60-day release) was implanted s.c. in each OVX mouse. Two weeks after surgery the mice were immunized with MBP. Serum E2 levels were quantified by ELISA. The composite means ± SD were calculated for at least 11 mice per group from four independent experiments. **p < 0.005 for comparisons to the SHAM controls (Mann-Whitney U test). B, Relationship between dietary vitamin D3 and serum E2 in MBP-immunized mice. The experiment was performed as in A. The serum E2 levels were quantified 24 days postsurgery (10 days postimmunization). The means ± SD (n = 6–8 mice/group) for one representative experiment of four are shown. **p < 0.005 and *p < 0.03 for the indicated comparisons (Mann-Whitney U test). C, Uterine weight. The experiment was performed as in A. The uterine weights were measured 4 wk after MBP immunization. The composite means ± SD for four independent experiments are shown (SHAM/−D/E2, n = 26; SHAM/−D/E2, n = 35; OVX/−D/E2, n = 29; OVX/−D/E2, n = 27; OVX/−D/E2, n = 12; OVX/−D/E2, n = 13). For statistical analysis, each OVX group was compared with the SHAM control group ingesting the same diet. **p < 0.01 and ***p < 0.001 (Mann-Whitney U test).
of MS often seen in men, and evaluated daily for EAE signs. The vitamin D$_3$-fed groups were not significantly different from the 0 μg/day vitamin D$_3$ controls for any EAE parameter (Table IV), consistent with our published data for male B10.PL mice (37). Implanting E$_2$ pellets in the males significantly increased their serum E$_2$ levels and inhibited EAE induction, as others reported previously (48). However, E$_2$ supplementation did not enable vitamin D$_3$ to further inhibit any EAE parameter in the males.

**Diestrus-level E$_2$ restored VDR gene expression and vitamin D$_3$ protection in OVX mice**

It is not clear why diestrus-level E$_2$ repletion restored the protective effects of vitamin D$_3$ in OVX females immunized to induce EAE. Two possibilities are E$_2$-mediated enhancement of in situ 1,25-(OH$_2$)D$_3$ accumulation through control of the Cyp27b1 or Cyp24a1 genes encoding the synthetic and inactivating enzymes, respectively, or E$_2$-mediated enhancement of the VDR in the CNS. The 1,25-(OH$_2$)D$_3$ synthesis and inactivation rates are proportional to the Cyp27b1 and Cyp24a1 transcripts, respectively (49). Therefore, to evaluate the propensity for hormone accumulation, we analyzed the Cyp27b1 and Cyp24a1 transcripts. As before, female B10.PL mice were fed +D or −D diets, OVX or SHAM surgery and placebo or E$_2$ pellet implantation was performed, and the mice were immunized with MBP. Ten days postimmunization (disease onset), spinal cord RNA was isolated and reverse transcribed, and transcripts were quantified by quantitative real-time PCR. Optimal PCR primers and reaction conditions were selected to amplify the transcripts with >90% efficiency (50). We recorded the threshold PCR cycles (Ct) at which the Cyp27b1 and Cyp24a1 amplicons were detected. The Ct is inversely proportional to transcript abundance, so the relative abundance of the two transcripts was expressed as ΔCt (Ct for Cyp24a1 minus the Ct for Cyp27b1) (Fig. 4A). In nonimmunized mice, the C$_t$ for Cyp24a1 was ~30 and the C$_t$ for Cyp27b1 was ~28–29, so ΔC$_t$ was ~1–2, regardless of dietary vitamin D$_3$. Assuming equal amplification efficiencies and fluorescence intensities, this ΔC$_t$ indicates that Cyp27b1 transcripts were slightly more abundant than Cyp24a1 transcripts. From standard curves we estimated that the Cyp27b1-to-Cyp24a1 transcript ratio was ~4:1 in naive mice.

In MBP-immunized SHAM and OVX mice, the C$_t$ for Cyp24a1 shifted to ~32–33, while the C$_t$ for Cyp27b1 remained ~28–29, so ΔC$_t$ was ~3–4, regardless of dietary vitamin D$_3$ (Fig. 4A). This increase in the C$_t$ for Cyp24a1 from ~30 in naive mice to ~32–33 in MBP-immunized mice indicates that Cyp24a1 gene expression decreased with MBP immunization, increasing the Cyp27b1-to-Cyp24a1 transcript ratio to ~8:1. These data are consistent with our previous report showing decreased Cyp24a1 gene expression during CNS inflammation in female mice (37). There was a trend toward further reduction in Cyp24a1 gene expression in OVX mice with E$_2$ implants that did not reach significance. Thus, the Cyp27b1-to-Cyp24a1 transcript ratio might be ~8:1 in these mice due to E$_2$-mediated Cyp24a1 suppression. Decreased Cyp24a1 gene expression during CNS inflammation would favor in situ 1,25-(OH$_2$)D$_3$ accumulation, consistent with the results presented in Table III.

We next investigated possible E$_2$-mediated enhancement of VDR gene expression in the CNS. Spinal cord samples collected 10 days postimmunization (disease onset) were analyzed for VDR and GAPDH transcripts. In samples from immununized controls, the C$_t$ for GAPDH and VDR were ~20 and ~29, respectively, regardless of dietary vitamin D$_3$. So, ΔC$_t$ (C$_t$ for VDR minus the C$_t$ for GAPDH) was ~9, indicating very low VDR gene expression (10–15 VDR copies/1000 GAPDH copies) (Fig. 4, B and C). In samples from the MBP-immunized SHAM mice ingesting the +D diet, the VDR C$_t$ decreased to ~24, indicating a ~32-fold increase in transcript abundance. This increase in VDR transcript abundance was also present in the MBP-immunized OVX mice with E$_2$ implants ingesting the +D diet, but it was absent in the −D SHAM group, the −D and +D OVX groups, and in the −D OVX group with E$_2$ implants. Thus, high-level VDR transcription in the spinal cord required an inflammatory stimulus, dietary vitamin D$_3$, and a source of E$_2$. Graphing the spinal cord VDR ΔC$_t$ at the time of EAE disease onset vs the cumulative EAE disease at the end of the 28-day study revealed a linear correlation between these parameters with an R$^2$ equal to 0.78 (Fig. 4D). This correlation was observed in two independent studies performed one year apart. We conclude that diestrus-level E$_2$ repletion restored the protective effects of vitamin D$_3$ in OVX females immunized to induce EAE, through decreased Cyp24a1 gene expression and increased VDR gene expression. These two actions enhanced in situ 1,25-(OH$_2$)D$_3$ accumulation and VDR function in the CNS, contributing to the resolution of inflammation and reduction of EAE disease.
One mechanism of synergy between vitamin D$_3$ and E$_2$ appears to be vitamin D$_3$ enhancement of E$_2$ biosynthesis. We found that vitamin D$_3$-supplemented OVX mice had 2-fold more serum E$_2$ than did unsupplemented mice. Our data are consistent with published data showing that VDR-targeted female mice had uterine hypoplasia and impaired folliculogenesis, because a lack of estrogen synthesis in the ovary decreased E$_2$ biosynthesis (51). E$_2$ supplementation reversed these defects. Moreover, 1,25-(OH)$_2$D$_3$ enhanced the transcription of the Cyp19 gene encoding estrogen synthase in glial cells (52) and placental trophoblasts (53). Estrogen synthase (also termed aromatase), the rate-limiting enzyme in the formation of estrone and estradiol from the C19 androgens androstenedione and testosterone, is expressed in the gonads, adrenals, brain, and adipose tissue (45). Thus, 1,25-(OH)$_2$D$_3$ and VDR-dependent enhancement of E$_2$ biosynthesis could be one mechanism allowing the estrogen and vitamin D$_3$ endocrine systems to function synergistically in women.

A second mechanism of synergy between the estrogen and vitamin D$_3$ endocrine systems appears to be E$_2$ suppression of Cyp24a1 gene expression, leading to 1,25-(OH)$_2$D$_3$ accumulation, and enhancement of VDR gene expression in females. Our new data show E$_2$-dependent transcriptional activation and function of the VDR gene in the spinal cord during an inflammation. This is true in other tissues as well. In osteoblasts, VDR gene expression decreased with E$_2$ deprivation and increased with E$_2$ supplementation (54, 55). Moreover, E$_2$-mediated up-regulation of the VDR gene was also reported in the duodenal mucosa (56), where reduced VDR gene methylation correlated with transcriptional activation, elevated VDR protein, increased responsiveness to endogenous 1,25-(OH)$_2$D$_3$, and greater resistance to colonic carcinogenesis (57, 58). Liver cells (59) and breast cells (60, 61) also showed E$_2$-mediated transcriptional activation of the VDR gene. In breast cells, an estrogen receptor (ER)-mediated mechanism controlled the VDR gene expression (60). Estrogen-responsive promoter elements were identified immediately upstream of exon 1c in the human VDR gene (61). Collectively, these data suggest that a second general mechanism of synergy between the estrogen and vitamin D$_3$ endocrine systems is E$_2$-mediated transcriptional activation of the VDR gene. Linking the first and second mechanisms together yields an amplification loop: 1,25-(OH)$_2$D$_3$

### Table II. $E_2$ repletion restored vitamin D$_3$-mediated inhibition of EAE in OVX female mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Surgery</th>
<th>Dietary Vitamin D$_3$ (μg/day)</th>
<th>$E_2$ Implant (mg)</th>
<th>Incidence (%)</th>
<th>Onset (day)</th>
<th>Peak Severity</th>
<th>Cumulative Disease Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.PL</td>
<td>SHAM</td>
<td>0</td>
<td>0</td>
<td>100 (25/25)</td>
<td>10 ± 3</td>
<td>2.3 ± 0.6</td>
<td>25.6 ± 9.2</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0</td>
<td>0</td>
<td>100 (30/30)</td>
<td>11 ± 4</td>
<td>2.0 ± 0.5</td>
<td>22.1 ± 11.5</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>1</td>
<td>0</td>
<td>100 (39/39)</td>
<td>11 ± 4</td>
<td>2.0 ± 1.0</td>
<td>20.9 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0.1</td>
<td>0.1</td>
<td>100 (25/25)</td>
<td>13 ± 9</td>
<td>2.1 ± 0.4</td>
<td>20.9 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>1</td>
<td>0.1</td>
<td>72 (18/25)</td>
<td>26 ± 10*</td>
<td>0.9 ± 0.2*</td>
<td>12.4 ± 4.9*</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>SHAM</td>
<td>0</td>
<td>0</td>
<td>100 (13/13)</td>
<td>15 ± 2</td>
<td>1.9 ± 0.7</td>
<td>20.9 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0</td>
<td>0</td>
<td>100 (13/13)</td>
<td>14 ± 1</td>
<td>1.7 ± 0.8</td>
<td>21.6 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>1</td>
<td>0</td>
<td>100 (13/13)</td>
<td>14 ± 1</td>
<td>2.4 ± 0.7</td>
<td>29.6 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0.1</td>
<td>0.1</td>
<td>100 (88/88)</td>
<td>18 ± 4</td>
<td>1.4 ± 0.8</td>
<td>13.8 ± 9.0*</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>1</td>
<td>0.1</td>
<td>86 (67)</td>
<td>19 ± 3</td>
<td>1.0 ± 0.6</td>
<td>8.7 ± 5.7*</td>
</tr>
</tbody>
</table>

a The experiment was performed as described in the Table I footnotes. The data are the composite means ± SD for two (C57BL/6) or four (B10.PL) independent experiments. The $^*$, $p < 0.05$ for comparisons between −D and +D mice within the same surgical and implant groups (Student’s t-test).

b Mice with a cumulative EAE severity score ≥1 were considered to have EAE. Numbers of mice are given parenthetically.

The day of onset was recorded as the day a mouse first had a cumulative EAE score ≥1.

d Each animal’s daily EAE disability scores were summed for 28 days postimmunization. #, $p < 0.02$ for the comparison between the $E_2$-repleted and placebo-repleted groups ingesting a diet without vitamin D$_3$. $^{*, ~p < 0.005}$ for the comparison between the $E_2$-repleted and placebo-repleted groups ingesting a diet with vitamin D$_3$.

### Table III. Spinal cord 1,25-(OH)$_2$D$_3$ in SHAM and OVX mice with placebo or $E_2$ implants and fed 0 or 1 μg/day vitamin D$_3$

<table>
<thead>
<tr>
<th>Surgery</th>
<th>Vitamin D$_3$ (μg/day)</th>
<th>$E_2$ Implant (mg)</th>
<th>Spinal Cord 1,25-(OH)$_2$D$_3$ (fmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>0</td>
<td>0</td>
<td>44 ± 24</td>
</tr>
<tr>
<td>SHAM</td>
<td>1</td>
<td>0</td>
<td>226 ± 293</td>
</tr>
<tr>
<td>OVX</td>
<td>0</td>
<td>0</td>
<td>59 ± 22</td>
</tr>
<tr>
<td>OVX</td>
<td>1</td>
<td>0</td>
<td>189 ± 64**</td>
</tr>
<tr>
<td>OVX</td>
<td>0.1</td>
<td>0.1</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>OVX</td>
<td>0.1</td>
<td>0.1</td>
<td>178 ± 92**</td>
</tr>
</tbody>
</table>

a Female B6 mice were fed diets with 0 or 1 μg/day vitamin D$_3$ continuously beginning 1 wk before surgery. OVX or SHAM surgery was performed and a placebo or $E_2$ pellet (0.1 mg; 60-day release) was implanted s.c. in each OVX mouse. Two weeks after surgery the mice were immunized with MOG35–55 peptide.

b Spinal cord 1,25-(OH)$_2$D$_3$ were quantitated at the end of the study. The data shown are the means ± SD (n=7 mice/group) from one experiment of three. **, $p < 0.005$ for comparisons between −D and +D mice within the same surgical and implant groups (Mann-Whitney test).

Discussion

We have presented evidence that female mice with disrupted ovarian hormone production lost vitamin D$_3$-mediated resistance to EAE and, conversely, that low-level $E_2$ repletion restored vitamin D$_3$-mediated resistance to EAE in OVX female mice. The low-level $E_2$ repletion did not inhibit EAE disease independently of dietary vitamin D$_3$. The vitamin D$_3$ in the +D diet used here was 3-fold higher than laboratory chow, and it yielded serum 25-(OH)D$_3$ levels that were ~1.6-fold higher than those in chow-fed mice. The vitamin D$_3$-mediated EAE resistance was manifested as a lower incidence, later onset, decreased peak severity, and diminished cumulative disease severity in the MBP/B10.PL model, and as a diminished cumulative disease severity in the MOG/B6 model. None of these vitamin D$_3$-mediated benefits were evident in male mice, with or without $E_2$ supplementation. Our data showed that high-level VDR gene expression and function in the spinal cord required an inflammatory stimulus, $E_2$, and sufficient dietary vitamin D$_3$ to support in situ 1,25-(OH)$_2$D$_3$ synthesis. To our knowledge, this is the first evidence of synergy between a sex hormone and vitamin D$_3$ in the control of an autoimmune disease, and the first evidence that $E_2$ is essential for VDR gene expression and function in the inflamed CNS.

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enables E2 biosynthesis by VDR-mediated up-regulation of estrogen synthase, and E2 enhances 1,25-(OH)2D3 synthesis and function by ER-mediated down-regulation of Cyp24a1 and up-regulation of VDR. These two mechanisms and possibly others allow the vitamin D3 and estrogen endocrine systems to function synergistically in women. Large increases in maternal serum estradiol and 1,25-(OH)2D3 levels occur during pregnancy due to placental production of these hormones (62). Thus, the synergy between the vitamin D3 and E2 endocrine systems may have evolved to support successful reproduction through maternal immune tolerance for fetal Ags.

The lack of interaction between E2 and vitamin D3 in males was puzzling, since males express ER, and E2 is an effective regulator of EAE in males (63). We suspect that there may be a differential regulation of the Cyp24a1 gene between males and females. We previously reported that males expressed higher levels of Cyp24a1 mRNA in the CNS than did females, and they did not show complete repression of Cyp24a1 gene expression during inflammation, as did females (37). This resulted in a failure to accumulate 1,25-(OH)2D3, which could undermine benefits derived from E2-mediated enhancement of VDR expression.

The synergy between 1,25-(OH)2D3 and E2 as inhibitors of EAE was necessary for E2 to inhibit EAE (73, 74). ER-expressing regulatory lymphocytes and antiinflammatory cytokines (66–69).

Finally, the two hormones have documented neuroprotective functions that involve regulation of neurotrophins and neurotrophin receptors (34, 70, 71). To explain how E2 enabled vitamin D3-mediated inhibition of EAE, we suggest that E2 and ER-α may enhance VDR expression in CD4+ T cells through estrogen-responsive promoter elements in the VDR gene (61).

An important question remaining to be answered is whether 1,25-(OH)2D3 exerts direct, E2-independent protective effects in EAE, or only indirect effects attributable to increased E2 synthesis. Similarly unanswered is the question whether E2 exerts direct, VDR-independent protective effects in EAE, or only indirect effects attributable to increased VDR synthesis. There are reported differences in the mechanisms of action of these two hormones that suggest independent action. For example, 1,25-(OH)2D3 reversed the signs of severe acute EAE (41), whereas E2 did not (48). Also, E2 inhibited EAE in mice with a targeted disruption of the IL-10 gene (72), whereas 1,25-(OH)2D3 did not (69). ER-α expression was necessary for E2 to inhibit EAE (73, 74).

\[ E2 \text{ supplementation did not enable vitamin D3 to inhibit EAE in intact male mice } \]

<table>
<thead>
<tr>
<th>Dietary Vitamin D3 (µg/day)</th>
<th>E2 Implant (mg)</th>
<th>Serum E2 (pg/ml)</th>
<th>Incidence (%)</th>
<th>Onset (day)</th>
<th>Peak Severity</th>
<th>Cumulative Disease Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>47 ± 9</td>
<td>100 (18/18)</td>
<td>17 ± 4</td>
<td>2.3 ± 1.0</td>
<td>20.6 ± 9.9</td>
</tr>
<tr>
<td>1</td>
<td>41 ± 22</td>
<td>100 (11/11)</td>
<td>19 ± 3</td>
<td>2.6 ± 1.4</td>
<td>18.1 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>ND</td>
<td>100 (5/5)</td>
<td>14 ± 1</td>
<td>2.2 ± 0.3</td>
<td>20.4 ± 7.1</td>
</tr>
<tr>
<td>0.18</td>
<td>480 ± 264*</td>
<td>87 (7/8)</td>
<td>23 ± 5*</td>
<td>1.2 ± 0.7</td>
<td>8.9 ± 5.0**</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>371 ± 279*</td>
<td>100 (7/7)</td>
<td>21 ± 4</td>
<td>1.5 ± 0.6</td>
<td>13.6 ± 6.9</td>
<td></td>
</tr>
</tbody>
</table>

*a* Male B6 mice were fed a diet with 0, 1, or 2 µg/day vitamin D3 continuously beginning 1 wk before the placebo or E2 pellets were implanted. Two weeks postimplantation, the mice were immunized with MOG35–55 peptide. The data are the composite means ± SD for two independent experiments. *p < 0.05 and **p < 0.01 for comparisons within diet groups.

*b* Serum E2 was quantified at the end of the 28-day EAE study. Shown are the means ± SD for n = 5–8 mice/group.

*c* Incidence, day of onset, and cumulative disability were defined and evaluated as described in the Table 2 footnotes.

\[ \text{FIGURE 4. The vitamin D3-mediated EAE resistance observed in SHAM females and E2-supplemented OVX females correlated with high level expression of VDR transcripts in the CNS.} \]

A. Relative Cyp24a1, and Cyp27b1 transcript abundance represented as ΔCt (Ct Cyp24a1 – Ct Cyp27b1). The experiment was performed as described in the Fig. 2 legend. Spinal cord samples were collected 10 days after MBP immunization. The RNA was isolated, reverse-transcribed, and real-time PCR was performed. The data are the means ± SD for one of two independent quantitative PCR analyses performed on samples from one of two independent experiments (n = 4–6 mice/group). For statistical analysis, the unprimed groups were compared with the SHAM MBP-primed groups. *p < 0.05 (Mann-Whitney U test).

B. VDR transcript abundance represented as ΔCt relative to GAPDH. C. VDR transcript abundance represented as copies per 1000 GAPDH copies. D. Correlation between cumulative EAE disease index and relative VDR transcript abundance represented as ΔCt relative to GAPDH.
neurons, glia, oligodendrocytes, lymphocytes, macrophages, and dendritic cells (34), but its expression in T lymphocytes was not necessary for E2-mediated inhibition of EAE (75). In contrast, our unpublished chimera data show that VDR expression is necessary in T lymphocytes for 1,25-(OH)2D3-mediated inhibition of EAE (C. G. Mayne, J. A. Spanier, and C. E. Hayes, unpublished data). These hormone receptor expression studies suggest that 1,25-(OH)2D3 may primarily target T lymphocytes, whereas E2 may primarily target non-T cells. Thus, although the two hormones E2 and 1,25-(OH)2D3 show many similar mechanisms of action suggesting synergy between them, they also show significant differences in mechanism suggesting each hormone can act independently. Additional experiments are underway to define the synergistic and independent functions of E2 and 1,25-(OH)2D3 more precisely.

The functional synergy between the vitamin D3 and E2 endocrine systems may be a driver for the relapsing-remitting MS disease phenotype. Seasonal fluctuations in UV light exposure and thus circulating 25-(OH)D3 levels show the same periodicity as seasonal fluctuations in MS attacks (15–21). The fact that seasonal changes in UV light/vitamin D3 preceded changes in MS attack rates by ~3–4 mo supports a causal link between UV light/vitamin D3 fluctuations and changes in the MS attack rates. Data showing beneficial effects of 25-(OH)D3 only in females is also consistent with this hypothesis (37, 38). Seasonal increases in UV light exposure and serum 25-(OH)D3 and spinal cord 1,25-(OH)2D3 levels could trigger the protective amplification loop described above. The increased E2 biosynthesis and E2-mediated enhancement of VDR expression and function would enable 1,25-(OH)2D3 and E2 to activate antiinflammatory mechanisms that drive MS disease into remission. Conversely, seasonal declines in UV light exposure and serum 25-(OH)D3 and spinal cord 1,25-(OH)2D3 levels could interfere with the amplification loop and allow inflammatory mechanisms to ignite and precipitate MS attacks.

The functional synergy between the vitamin D3 and E2 endocrine systems may also be a driver of the increasing female bias in MS. An equal number of women and men were afflicted with MS. An equal number of women and men were afflicted with MS, establishing a female gender bias. Moreover, the lifestyle changes that have decreased overall UV light exposure and serum 25-(OH)D3 levels in women over the last half century (83) could be driving the rapidly increasing female gender bias. Examples of significant lifestyle changes are increased numbers of women in the workforce, decreased outdoor activity, increased sun avoidance, and use of sunscreens (which inhibit vitamin D3 biosynthesis). We suggest that the vitamin D3 and E2 functional synergy hypothesis, rather than the X-chromosome or sex hormone hypothesis, is most consistent with recent data on the rapid rise in MS among women.

We have proposed that there is functional synergy between the vitamin D3 and E2 endocrine systems, and that this synergy is causally related to the relapsing-remitting MS disease phenotype and the increasing female bias in MS prevalence. Our data and the mechanisms we have proposed to explain the data have very significant implications for MS. Specifically, inadequate sunlight exposure and low vitamin D3 supplies may undermine the beneficial effects of estrogens, to the extent that these activities depend on enhancement of VDR expression and function. Moreover, inadequate E2 biosynthesis due to ovarian failure or menopause may undermine the beneficial effects of vitamin D3, to the extent that these activities depend on enhancement of E2 synthesis. Combined vitamin D3 and E2 deprivation, such as one might expect for older female MS patients with limited mobility, could have a devastating synergistic effect, triggering the evolution of relapsing-remitting MS into a chronic-progressive disease course with rapid accumulation of disability.

MS is a devastating neurodegenerative disease that imposes heavy burdens on patients, on families, and on health care systems throughout the world. At an estimated lifetime cost in excess of $2.2 million per MS case, the implications of the sustained increases in female cases to the world’s strained health care systems are staggering (28). In this context, it is encouraging that modifiable environmental factors appear to set the disease threshold and may hold the key to preventing the vast majority of MS cases (6). Sunlight exposure and vitamin D3 supplies appear to be those modifiable environmental risk factors (9, 13). If health care providers were to monitor serum 25-(OH)D3 levels, especially in girls and women who are genetically related to an individual with MS, and prescribe enough sunlight exposure and/or vitamin D3 supplementation to maintain >100 nmol/L of serum 25-(OH)D3 throughout the year, an estimated 90% of MS cases might be prevented (15). For men and women already afflicted with MS, intermittent 1,25-(OH)2D3 pulse dose therapy (F. E. Nashold, R. A. Derks, and C. E. Hayes, manuscript in preparation) in the context of sufficient natural E2 in young women or E2 replacement therapy in postmenopausal women might activate antiinflammatory mechanisms that drive MS disease into remission and significantly decrease the cumulative disability. The overwhelming body of evidence suggests that these intervention strategies could dramatically reduce the impact of MS on patients, on families, and on our health care systems.

Acknowledgments

We thank Dr. Halina Offner for discussion on the research and critically reading the manuscript. We acknowledge Anna R. Raines for technical assistance, Dr. Peter Crump for statistical advice, and the Biochemistry Animal Care staff for careful animal husbandry.
Disclosures

The authors have no financial conflicts of interest.

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is associated with decreased CpG island methylation and increased mRNA and protein expression of the colonic vitamin D receptor. Oncol. Res. 11: 255–264.


