Immune Modulation in Multiple Sclerosis Patients Treated with the Pregnancy Hormone Estriol[1]

Samantha S. Soldan, Ana Isabel Alvarez Retuerto, Nancy L. Sicotte, and Rhonda R. Voskuhl[2]

The protective effect of pregnancy on putative Th1-mediated autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, is associated with a Th1 to Th2 immune shift during pregnancy. The hormone estriol increases during pregnancy and has been shown to ameliorate experimental autoimmune encephalomyelitis and collagen-induced arthritis. In addition, estrogens induce cytokine changes consistent with a Th1 to Th2 shift when administered in vitro to human immune cells and in vivo to mice. In a pilot trial, oral estriol treatment of relapsing remitting multiple sclerosis patients caused significant decreases in enhancing lesions on brain magnetic resonance imaging. Here, the immunomodulatory effects of oral estriol therapy were assessed. PBMCs collected longitudinally during the trial were stimulated with mitogens, recall Ags, and glatiramer acetate. Cytokine profiles of stimulated PBMCs were determined by intracellular cytokine staining (IL-5, IL-10, IL-12 p40, TNF-α, and IFN-γ) and cytometric bead array (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ). Significantly increased levels of IL-5 and IL-10 and decreased TNF-α were observed in stimulated PBMC isolated during estriol treatment. These changes in cytokines correlated with reductions of enhancing lesions on magnetic resonance imaging in relapsing remitting multiple sclerosis. The increase in IL-5 was primarily due to an increase in CD4$^+$ and CD8$^+$ T cells, the increase in IL-10 was primarily due to an increase in CD64$^+$ monocytes/macrophages with some effect in T cells, while the decrease in TNF-α was primarily due to a decrease in CD8$^+$ T cells. Further study of oral estriol therapy is warranted in Th1-mediated autoimmune diseases with known improvement during pregnancy. ***The Journal of Immunology*****, 2003, 171: 6267–6274.**

Multiple sclerosis (MS)[3] is the most prevalent demyelinating disease of the CNS, affecting an estimated 1,100,000 individuals worldwide (1). While the etiology of MS is unknown, a Th1-mediated autoimmune component driven by myelin protein-specific, proinflammatory cytokine-secreting T lymphocytes is believed to be involved in the pathogenesis. A beneficial effect of pregnancy on clinical symptoms has been observed in MS and other Th1-mediated autoimmune diseases, including rheumatoid arthritis (RA), psoriasis, uveitis, and thyroiditis (2–20). Interestingly, most of these diseases are characterized by temporary rebound exacerbations postpartum (2, 4, 5, 7–9, 14, 17, 20). Specifically in MS, the improvement in clinical symptoms during pregnancy entails a significant reduction in relapse rates in the last trimester, and the postpartum exacerbation involves a temporary increase in relapse rates to levels higher than prepregnancy levels for ~6 mo. In contrast, a worsening of symptoms during pregnancy has been reported in systemic lupus erythematosus, an autoimmune disease with a putative Th2-mediated component in its pathogenesis (21, 22).

In general, Th1 lymphocytes secrete proinflammatory cytokines (e.g., IL-2, IL-12, IFN-γ, and TNF-α) that promote cellular immunity, while Th2 lymphocytes produce anti-inflammatory cytokines (e.g., IL-4, IL-5, IL-6, and IL-10) that promote humoral immunity. Th2 cytokines are associated with the down-regulation of Th1 cytokines and may confer protection from Th1-mediated autoimmune diseases. During pregnancy, there is a shift from Th1 to Th2 that occurs both locally, at the fetal maternal interface, (23–25), and systemically (26–31). This immune shift is thought to be necessary to avoid fetal rejection (25, 29, 32, 33), since failure to achieve a Th1 to Th2 immune deviation has been associated with increased risk of spontaneous abortion (29, 31, 34). This naturally occurring, systemic shift in immune responses may underlie improvements in Th1-mediated autoimmune diseases during pregnancy.

A variety of pregnancy factors may be responsible for the immune shift during pregnancy, including sex hormones, vitamin D, α-fetoprotein, early pregnancy factor, human chorionic gonadotropin, and pregnancy-specific glycoproteins (35). The hormone estriol is an estrogen made by the fetal placental unit, and hence only becomes detectable when women are pregnant. Estriol levels increase progressively throughout pregnancy, peaking during the third trimester when the beneficial effects of pregnancy in relapsing remitting MS (RRMS) are most profound. Also, estriol levels drop precipitously postpartum when relapse rates rebound (7). Thus, there is a direct association of increased estriol levels with disease protection in RRMS.

Support for estriol as a candidate pregnancy factor that may contribute to the amelioration of Th1-mediated autoimmune diseases is derived from in vitro studies of T cells derived from humans and in vivo studies of experimental autoimmune diseases in mice. T cell lines and purified T cells have each been shown to switch their cytokine profile from Th1 to Th2 when cultured with

Department of Neurology, Reed Neurological Research Center, University of California School of Medicine, Los Angeles, CA 90095

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[2] Address correspondence and reprint requests to Dr. Rhonda R. Voskuhl, Reed Neurological Research Center, Room A145, 710 Westwood Plaza, Los Angeles, CA 90095. E-mail address: rvoskuhl@ucla.edu

[3] Abbreviations used in this paper: MS, multiple sclerosis; CBA, cytometric bead array; EAE, experimental autoimmune encephalomyelitis; EDSS, Expanded Disability Status Scale; MMP-9, matrix metalloproteinase-9; MRI, magnetic resonance imaging; RA, rheumatoid arthritis; RRMS, relapsing remitting MS; SPMS, secondary progressive MS; COP-1, copolymer-1.

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pregnancy levels of estriol (36, 37). In addition, estriol has been shown to ameliorate experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis when administered in vivo at pregnancy levels (38–40). Moreover, shifts in cytokine profiles of immune cells from estrogen-treated EAE mice have supported the concept that at least one mechanism of estrogen's protection in these diseases involves a favorable alteration in cytokine production (38, 40, 41).

Collectively, the protective effect of pregnancy in MS and EAE and the anti-inflammatory effects of estriol have suggested that hormones associated with pregnancy, estriol in particular, may exert a beneficial influence on MS. Accordingly, a pilot clinical trial using oral estriol to treat 10 female MS patients (six with RRMS and four with secondary progressive MS (SPMS)) was completed (42). A cross-over design was used in which a pretreatment baseline was followed by a treatment period. Post-treatment and retreatment phases were also examined. During oral estriol therapy, RRMS patients demonstrated significantly decreased gadolinium-enhancing lesions on monthly cerebral magnetic resonance imaging (MRI), decreased delayed-type hypersensitivity responses to tetanus, and decreased IFN-γ message in unstimulated PBMC (42). Given these encouraging findings, it was of interest to determine whether the beneficial effect of oral estriol treatment observed in this small cohort of RR patients was associated with changes in cytokine profiles consistent with an anti-inflammatory, Th1 to Th2, shift in stimulated PBMCs. To our knowledge this is the first time alterations in cytokine production of stimulated PBMCs have been identified when an estrogen has been given in vivo to patients with an autoimmune disease.

Materials and Methods

Oral estriol trial design and patients

Ten female patients with clinically definite MS (six RR and four SP) completed the study. The patients had not received steroid treatment for at least 3 mo or IFN-β or glatiramer acetate for 6 mo before participating in the study. Patients who were pregnant, nursing, taking oral contraceptives, or receiving hormone replacement therapy were excluded. The mean age was 44 years (range, 28–50 years). The mean Expanded Disability Status Scale (EDSS) score was 3.3 (range, 1.0–6.5), with a clear differences between RRMS and SPMS groups (mean EDSS of RRMS = 2.2; mean EDSS of SPMS = 5.0). The cross-over design consisted of a 6-mo pretreatment period, a 6-mo oral estriol treatment period (8 mg/day), and a 6-mo post-treatment period with brain MRIs monthly and clinic visits with blood sample collection every 3 mo. In the RRMS cohort, the trial was extended by a 4-mo retreatment period. Oral estriol was tapered over 2 wk after the treatment period to avoid a precipitous drop in hormone levels. During the retreatment period, oral estriol was used in combination with progesterone (100 mg/day) to protect against endometrial hyperplasia. Estriol levels during treatment were shown to approximate those that occur naturally at 6 mo of pregnancy (42). The study was approved by the University of California-Los Angeles human subjects protection committee, and informed consent was obtained from all patients enrolled in the trial.

Cell proliferation assay

PBMCs were isolated by a conventional Ficoll-Hypaque method from freshly drawn blood specimens and cryopreserved. PBMCs from all time points from a given patient were assayed in parallel on the same day in the following manner. Cryopreserved PBMC were thawed and cultured in triplicate on a 96-well, round-bottom tissue culture plate at 1×10^5 cells/well in X-Vivo 20 serum-free medium (BioWhittaker, Walkersville, MD) supplemented with 1× penicillin/streptomycin and 2 mM L-glutamine (BioWhittaker), either alone or with anti-CD3 (1 μg/ml; Sigma-Aldrich, St. Louis, MO), PHA (5 μg/ml; Sigma-Aldrich), glatiramer acetate/copolymer 1 (COP-1) (10 μg/ml; Teva, North Wales, PA), tetanus toxoid (10 LFA/ml; Canaught Laboratories, Swiftwater, PA), or *Candida albicans* lysate (CA) (40 μg/ml). Cells were plated with medium alone or with test Ags in triplicate. Three identical plates were prepared for harvest on days 2, 4, and 6. Cultured PBMCs were pulsed with 1 μCi/well of [*methyl*-^{3}H]thymidine for the final 18 h of incubation. Cells were harvested with a cell harvester, and tritiated thymidine incorporation was measured by a liquid scintillation counter (Wallac, Gaithersburg, MD). Means and SDs were assessed from triplicate wells. Stimulation indexes were calculated by dividing the counts per minute in stimulated wells by the counts per minute in unstimulated wells.

Analysis of secreted cytokines by cytometric bead array

Cryopreserved PBMC were thawed and cultured in a 96-well, round-bottom tissue culture plate at 1×10^5 cells/well for 48 h with media and Ags as described above for proliferation assays. Supernatants were harvested and stored at −70°C until cytokine testing was performed. IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were detected simultaneously using the human Th1/Th2 cytokine cytometric bead array (CBA) kit (BD PharMingen, San Diego, CA). Briefly, 50 μl of each sample was mixed with 50 μl of mixed capture beads and 50 μl of the human Th1/Th2 PE detection reagent consisting of PE-conjugated anti-human IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ. The samples were incubated at room temperature for 3 h in the dark. After incubation with the PE detection reagent, the samples were washed once and resuspended in 300 μl of wash buffer before acquisition on the FACSCalibur (BD Biosciences, Sunnyvale, CA). Data were analyzed using CBA software (BD PharMingen). Standard curves were generated for each cytokine using the mixed cytokine standard provided by the kit. The concentration for each cytokine in cell supernatants was determined by interpolation from the corresponding standard curve. The range of detection was 20–5000 pg/ml for each cytokine measured by CBA.

Intracellular cytokine and surface marker staining

Cryopreserved PBMC from all time points of a given patient were thawed in parallel. One aliquot of cells was stained for cell surface markers with a panel of FITC-conjugated Abs, including CD4, CD8, CD19, CD64, CD27, CD54 (ICAM), and CD106 (VCAM), or with Cy5-conjugated Abs (CD45 Ra, CD45 Ro, CD80, CD86, and CD25). The other aliquot of cells was cultured in a 96-well, round-bottom tissue culture plate at 1×10^5 cells/well with media and Ags as described above for 24 h. Brefeldin A was added during the last 5 h of stimulation. Cells were washed in staining medium (1× PBS, 2% FCS, and 0.1% NaN_3) and stained with the same panel of FITC-conjugated Abs (CD4, CD8, CD19, CD64, CD27, CD54, and CD106) and Cy5-conjugated (CD45Ra, CD45Ro, CD80, CD86, and CD25) for 45 min at 4°C. Cells were washed twice with staining medium and fixed and permeabilized with Cytofix/Cytoperm solution (BD PharMingen). Cells were then resuspended in perm/wash buffer (BD PharMingen) and stained with PE-labeled Abs specific for IL-5, IL-10, IL-12p40, TNF-α, and IFN-γ (BD PharMingen) for 20 min at room temperature. After intracellular cytokine staining, cells were washed twice in perm/wash buffer and resuspended in 400 μl of staining medium before three-color FACS analysis on a FACSCalibur instrument (BD Biosciences) using CellQuest software (BD Biosciences). For each experiment cells were also stained with isotype control Abs to establish background staining and to set quadrants before calculation of the percentage of positive cells.

Results

During treatment with the pregnancy hormone estriol, we had previously observed a decrease in the delayed-type hypersensitivity response to a recall Ag, a decrease in IFN-γ message in unstimulated PBMCs, and a decrease in gadolinium-enhancing lesions on brain MRI (42). Since pregnancy had previously been associated with changes in cytokine production by stimulated immune cells and also with changes in subpopulations of circulating immune cells (40–42), we examined whether these changes also occurred in the above MS patients treated with estriol.

Alterations in subpopulations of circulating PBMCs during oral estriol treatment

PBMCs obtained during pretreatment, treatment, post-treatment, or retreatment phases of the estriol trial were stained ex vivo for cell surface marker expression (no in vitro culture). Subpopulations of circulating immune cells were altered during in vivo estriol treatment in both RRMS and SPMS groups; however, changes were slightly more robust in the RRMS group than in the SPMS group (Fig. 1, *A* and *B*). Oral estriol treatment was associated with significant decreases in $CD4^+$ and $CD8^+$ T cells and an increase in $CD19^+$ B cells, with no changes in $CD64^+$ monocytes/macrophages. Significant decreases in $CD4^+CD45Ro^+$ (memory T

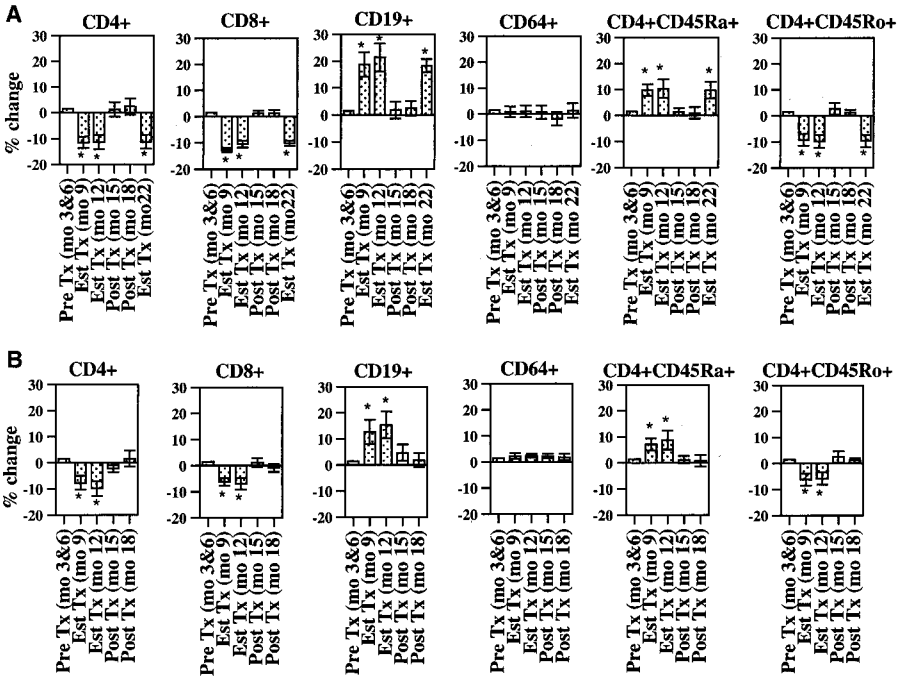

FIGURE 1. Subpopulations of circulating PBMCs from MS patients were altered by oral estriol treatment. PBMCs from RRMS (*A*) and SPMS (*B*) patients acquired during the estriol trial were stained ex vivo for cell surface marker expression (no in vitro culture). RRMS included pretreatment (mean of mo 3 and 6), estriol treatment (mo 9 and 12), post-treatment (mo 15 and 18), and retreatment (mo 22), while SPMS included pretreatment, treatment, and post-treatment only. Data are expressed as the percent change from the mean of the two baseline pretreatment samples (mo 3 and 6). Error bars represent variability (SEM) between patients within each group. *, $p < 0.005$, by Wilcoxon/Kruskal-Wallis rank-sum analysis. Oral estriol was associated with decreases in $CD4^+$ and $CD8^+$ T cells and an increase in $CD19^+$ B cells with no changes in $CD64^+$ monocytes/macrophages. Also, there were decreases in $CD4^+CD45Ro^+$ (memory T cells) and increases in $CD4^+CD45Ra^+$ (naive T cells). While significant changes occurred in both RRMS and SPMS, they were occasionally more robust in the RRMS group. Pre Tx, pretreatment; Est Tx, estriol treatment; Post Tx, post-treatment; Est Tx, estriol retreatment in combination with progesterone.

cells) and increases in $CD4^+CD45Ra^+$ (naive T cells) were also observed. There was a slight trend for decreases in $CD4^+CD25^+$ cells during treatment as well, but this did not reach significance, and no changes in the percentages of $CD80^+$, $CD86^+$, $CD54^+$, or $CD106^+$ cells were found (data not shown). During the post-treatment phase, levels of circulating cells returned to baseline pretreatment levels. During the retreatment phase, which took place only in RRMS patients, changes similar to the treatment phase reoccurred.

In vivo estriol treatment has no effect on proliferative responses

Lymphoproliferative responses to α-CD3, *C. albicans*, PHA, COP-1, tetanus toxoid, and *C. albicans* were assessed throughout the trial. The majority of MS patients had positive lymphoproliferative responses (stimulation index, >2) to α-CD3 (six of six RR; four of four SP), PHA (six of six RR, four of four SP), and COP-1 (six of six RR; four of six SP). Fewer patients had positive responses to *C. albicans* lysate (four of six RR, three of four SP) and tetanus toxoid (two of six RR, zero of six SP). No significant difference in lymphoproliferative response to any of the test Ags was demonstrated during oral estriol therapy (data not shown).

In vivo estriol treatment alters secreted cytokines from stimulated PBMCs

PBMCs from were stimulated with anti-CD3, glatiramer acetate, recall Ags (tetanus toxoid and *C. albicans* lysate), and PHA. Cytokine secretion (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) was measured from supernatants of samples with positive lymphoproliferative responses (stimulation index, >2 to a given Ag) by CBA. Data were analyzed as the mean percent change during the indicated treatment time point compared with the mean of two pretreatment baseline values. As demonstrated in Fig. 2, upon stimulation with αCD3, PHA, and *Candida*, IL-5 and IL-10 secretion were significantly increased, and TNF-α levels were significantly decreased in both RRMS and SPMS during the initial oral estriol treatment phase (mo 9 and 12) compared with pretreatment baseline values (mean of mo 3 and 6). During the post-treatment phase (mo 15 and 18), levels of secreted IL-5, IL-10, and TNF-α returned to baseline in both patient groups. Further, in the retreatment phase (mo 22) of the RRMS group, levels of IL-5 and IL-10 were again increased, and TNF-α was again decreased (Fig. 2*A*). Oral estriol treatment did not have a statistically significant effect on IL-2, IL-4, or IFN-γ. There was, however, a trend that did not reach significance for decreased IFN-γ during treatment in the RRMS group. Similar changes in cytokine profiles were observed in supernatants from PBMC with positive lymphoproliferative responses to tetanus toxoid and COP-1 (data not shown).

In vivo estriol treatment alters intracellular cytokine production from stimulated PBMCs

To determine whether estriol affected cytokine production and as a complement to secreted cytokine determination in supernatants, intracellular production of IL-5, IL-10, IL-12p40, TNF-α, and IFN-γ was measured by FACS analysis after 24 h of stimulation with the same test Ags. As demonstrated in Fig. 3, intracellular IL-5 and IL-10 were again significantly increased, while TNF-α was again significantly decreased during treatment (mo 9 and 12), with changes generally more robust in the RRMS group (Fig. 3*A*) than in the SPMS group (Fig. 3*B*). Intracellular cytokine levels again returned to baseline during the post-treatment period (mo 15 and 18) in both MS groups. In the retreatment phase (mo 22) in the RRMS group, increased IL-5 and IL-10 with decreased TNF-α recurred. Oral estriol treatment did not have a statistically significant effect on intracellular IL-12 p40 or IFN-γ. Similar changes in cytokine profiles were demonstrated after stimulation with tetanus toxoid and COP-1 (data not shown).

Correlation between enhancing lesion volumes and changes in cytokine profiles during oral estriol therapy in RRMS

Significant decreases in enhancing lesion volume and number were observed with oral estriol treatment (mo 7–12) compared with pretreatment baseline (mo 1–6) on monthly MRI in the RRMS, but not the SPMS, group (42). In the RRMS group, lesion volumes and numbers increased to pretreatment baseline after treatment was

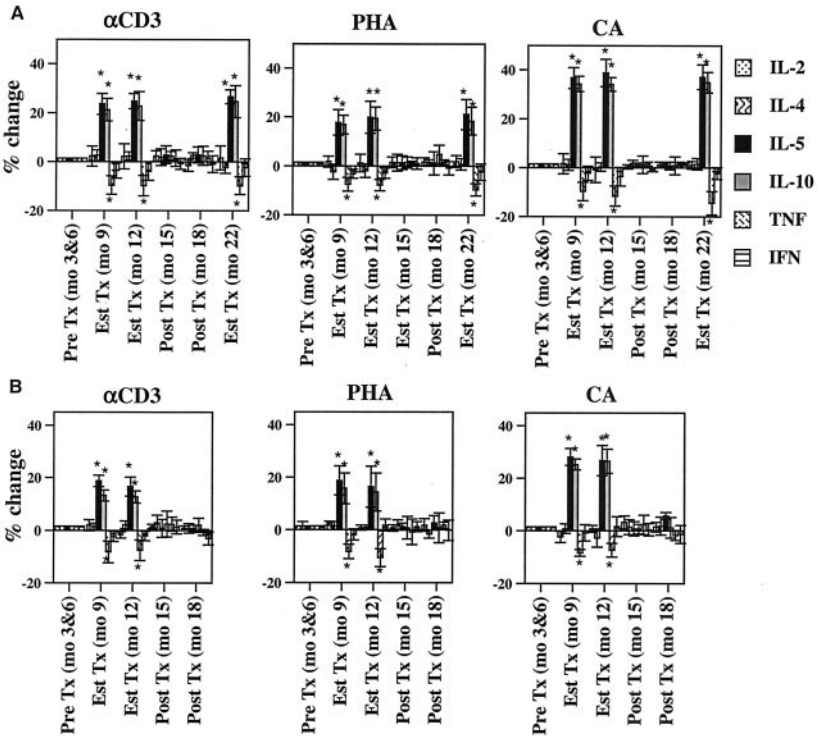

FIGURE 2. In vivo estriol treatment increased secreted IL-5 and IL-10 and decreased secreted TNF-α from stimulated PBMCs. Secreted cytokine levels (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) were assessed in culture supernatants 48 h after stimulation by CBA. PBMC from RRMS (*A*; $n = 6$) and SPMS (*B*; $n = 4$) were stimulated with αCD3, PHA, or *C. albicans* lysate (CA). Cytokine levels are expressed as the mean percent change of the level in the samples at the indicated treatment time point compared with the mean from two pretreatment baseline time points (mo 3 and 6), with error bars indicating the SEs between patients within each group. $*$, $p < 0.005$, by Wilcoxon/Kruskal-Wallis rank-sum analysis). The range of detection was 20–5000 pg/ml for each cytokine.

stopped (mo 13–18), then again significantly decreased with reinstitution of oral estriol therapy (mo 19–22). Therefore, it was of interest to determine whether these MRI findings, representing a clinical measure of disease activity, correlated with the changes in secreted cytokine profiles observed during oral estriol therapy. As demonstrated in Fig. 4, there was an inverse correlation between αCD3-stimulated levels of IL-5 and IL-10 with mean enhancing lesion volumes, while there was a direct correlation between TNF-α levels and enhancing lesions (cubic millimeters) in the RRMS group. Similar results were observed in PBMC from RRMS stimulated with PHA, COP-1, and *C. albicans* lysate (data not shown). Gadolinium-positive lesion volumes per scan were lower in the SPMS group (mean, 18.9; median, 0 mm^3) than in the RRMS group (mean, 41.9; median, 19.5 mm^3), and the above correlations between MRI and cytokine levels observed in the RRMS group were not observed in the SPMS group.

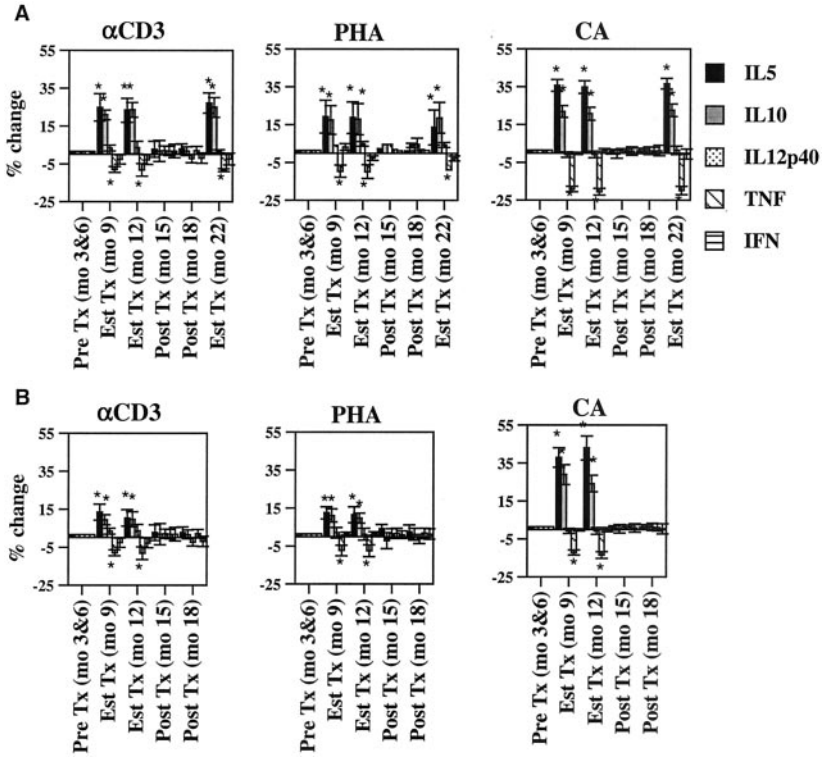

FIGURE 3. In vivo estriol treatment increased intracellular IL-5 and IL-10 and decreased intracellular TNF-α from stimulated PBMCs. Intracellular cytokine levels (IL-5, IL-10, IL-12p40, TNF-α, and IFN-γ) were measured in PBMC from patients in the RRMS group (*A*) and the SPMS group (*B*) following 24-h stimulation with α-CD3, PHA, and *C. albicans* lysate (CA). Levels of cells with positive cytokine staining after subtracting staining observed with negative control Ab were determined at each time point and expressed as the percent change from baseline as in Fig. 2. Error bars indicate the SEs between patients within each group. $*$, $p < 0.005$, by Wilcoxon/Kruskal-Wallis rank-sum analysis.

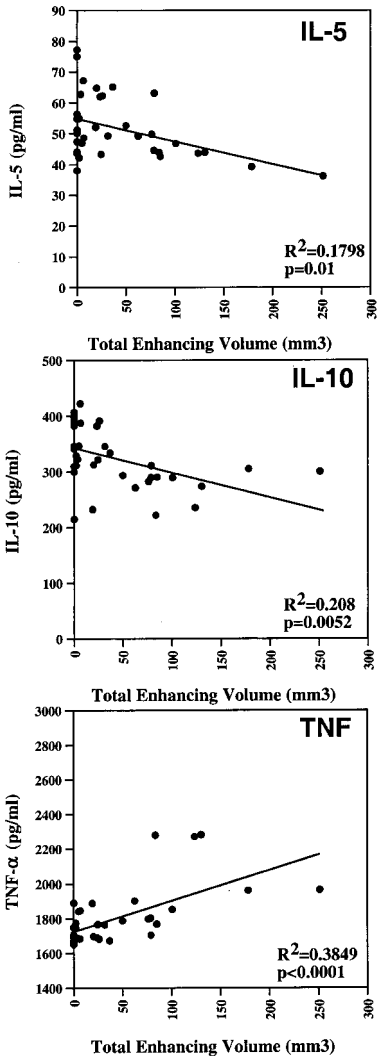

FIGURE 4. Brain MRI enhancing lesions in RRMS patients correlate inversely with IL-5 and IL-10 and directly with TNF-α. Type I regression analysis of gadolinium-enhancing lesion volumes (cubic millimeters) and secreted cytokine levels (picograms per milliliter) after αCD3 stimulation of PBMCs from RRMS patients.

Identification of cell phenotypes affected by in vivo estriol treatment

Three-color flow cytometry with cell surface marker-specific Abs were used to determine which cells demonstrated the altered cytokine production during in vivo estriol treatment. As shown in Fig. 5, after stimulation with αCD3, the increase in IL-5 was primarily due to increases in $CD4^+$ and $CD8^+$ T cells. The increase in IL-10 during treatment was primarily due to an increase in $CD64^+$ cells (macrophages), with a smaller increase in T cells. The decrease observed in TNF-α was primarily due to a decrease in $CD8^+$ T cells, with a slight decrease in $CD4^+$ T cells. No change in any of these cytokines was observed in the $CD19^+$ population (B cells). Cell phenotype-specific changes in cytokine profiles were similar in RRMS (Fig. 5*A*) and SPMS (Fig. 5*B*), but were generally more robust in the RRMS group. Similar results were obtained for PBMC stimulated with PHA, *C. albicans* lysate, and COP-1.

Discussion

A partial Th1 to Th2 shift was observed in stimulated PBMCs from MS patients during in vivo estriol treatment, which consisted of increased production of IL-5 and IL-10 and decreased TNF-α regardless of whether a mitogen or recall Ag was used for stimulation. Cytokine profiles of $CD4^+$ and $CD8^+$ T cells and $CD64^+$ monocytes/macrophages were all significantly affected by in vivo estriol treatment, while cytokine production by B cells was unaffected. Of interest, increased levels of IL-5 and IL-10 and decreased TNF-α production correlated with the mean volume of enhancing lesions on MRI in the RRMS group. This correlation between changes in cytokine profiles and lesion volume suggests that the anti-inflammatory effects of estriol may have an effect on disease in the target organ. The partial Th1 to Th2 immune shift observed in female MS patients treated with in vivo estriol is consistent with the general shift away from a Th1-biased response observed in males receiving in vivo ethinyl estradiol and an antiandrogen for gender reassignment (43). To our knowledge, our data are the first to describe cytokine changes induced by administration of an estrogen alone in patients with an autoimmune disease. These results are in agreement with previous in vitro studies of human immune cells and in vivo studies of mice that demonstrated an anti-inflammatory role for estriol. Specifically, increased expression of IL-10 and decreased TNF-α were reported in human T cell lines and in unprimed T cells cultured in vitro in the presence of estriol (36, 37). Further, in vivo estriol treatment was shown to ameliorate both collagen-induced arthritis and EAE (38–40, 44). In adoptive EAE, estriol treatment increased IL-10 production by splenocytes stimulated with autoantigen and increased autoantigen-specific IgG1 Ab production consistent with an up-regulation of Th2 responses (38), while in active EAE, estriol treatment decreased TNF-α and IFN-γ production by lymph node cells stimulated with autoantigen, consistent with a down-regulation of Th1 responses (40, 41).

Since an alteration in circulating immune cells could theoretically influence cytokine production upon stimulation of PBMCs ex vivo, and since an alteration in circulating immune cells has been previously reported during pregnancy (45–47), we ascertained whether an alteration in circulating immune cells occurred during in vivo treatment with the pregnancy hormone estriol. In addition to an alteration in cytokine production by immune cells, subpopulations of circulating immune cells were indeed significantly altered during in vivo estriol treatment. In both RRMS and SPMS, there were significant decreases in $CD4^+$ and $CD8^+$ T cells and increases in B cells. Further, there were significant decreases in $CD4^+CD45Ro^+$ (memory T cells) and increases in $CD4^+$ $CD45Ra^+$ (naive T cells). Together these data indicate that in vivo estriol treatment at pregnancy doses not only alters cytokine production from T cells and monocytes/macrophages, but also alters the repertoire of circulating cell types. Our findings of alterations in both cytokine production and circulating immune cell subpopulations have relevance to estrogen administration to patients with autoimmune diseases.

In addition to effects on cytokine production and on the repertoire of circulating immune cells, estriol may work through additional immune mechanisms. Recently, estriol has been shown to reduce trafficking of immune cells by inhibiting matrix metalloproteinase-9 (MMP-9) expression via down-regulation of the transcription factor NF-κB (37). MMP-9, an inducible form of MMP, is elevated in acute MS lesions and is thought to be important in T cell migration into the target organ in MS (48). Estriol may also operate through other immune molecules regulated by NF-κB, including several cytokines, chemokines, adhesion molecules, MHC molecules, and NO synthase. For example, the adhesion molecules ICAM and VCAM are induced by NF-κB, and their expression may also be down-regulated by estriol (49). While there was no

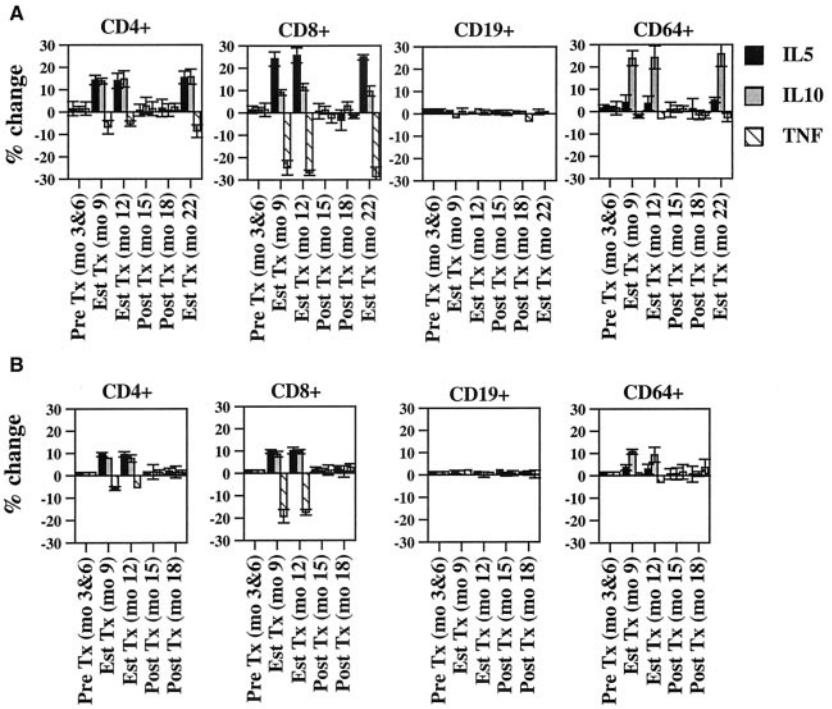

FIGURE 5. Cell phenotype-specific effects of in vivo estriol treatment on cytokine profiles. Intracellular cytokine levels in αCD3-stimulated PBMCs double-stained for cell surface markers ($CD4^+$ T cell, $CD8^+$ T cell, $CD19^+$ B cell, $CD64^+$ monocytes/macrophage) and intracellular cytokines in RRMS (*A*) and SPMS (*B*). The increase in IL-5 was primarily due to an increase in $CD4^+$ and $CD8^+$ T cells, the increase in IL-10 was primarily due to an increase in $CD64^+$ macrophages, with some effect in T cells, while the decrease in TNF-α was primarily due to a decrease in $CD8^+$ T cells. Responses to treatment were generally more robust in RRMS than in SPMS. Cytokine levels are expressed as the mean percent change at the indicated time point compared with the mean of the two pretreatment baseline samples (mo 3 and 6). Error bars represent variability (SEM) between patients within each group.

significant effect of estriol treatment on VCAM or ICAM expression in PBMC from the relatively small cohort of patients examined in this study (data not shown), a direct influence of estriol on adhesion molecules at the blood-brain barrier could influence CNS permeability and disease activity. In MS, estriol may also work through additional mechanisms within the target organ, ranging from decreased microglial activation to protection from neuronal apoptosis (50–54).

The majority of MS patients have either RRMS or SPMS, representing a continuum of disease progression over decades. The early RR phase is characterized by frequent relapses and relatively little disability, with an MRI correlate of inflammation (enhancing lesions) and relatively less neuronal cell loss (atrophy and "T1 holes"). In contrast, the late SP phase is characterized by progressive disability in the absence of acute relapses, less inflammation, and more neuronal cell loss on MRI. The approved injectable treatments (the IFN-β drugs and copaxone) have all been shown to be of proven benefit in the early RRMS, but not the late SPMS. This is consistent with the fact that all of the above treatments are anti-inflammatory. It was interesting to compare the effects of estriol on the two distinct groups in our study (RRMS and late SPMS). In our previous study the SPMS group differed from the RRMS group in both the lack of a treatment effect on enhancing lesions on MRI and the lack of an effect on IFN-γ message levels in unstimulated PBMCs. This was consistent with the trend for a less robust effect of estriol treatment on cytokine production by mitogen and Ag-stimulated immune cells in SPMS compared with RRMS in this study. Together these data support observations that the immune dysregulation in RRMS and SPMS may differ and that the late SPMS group may ultimately become more refractory to some immunomodulatory therapies (55–59). Thus, the refractory nature of the SPMS stage may reside not only in the relatively greater contribution of neuronal cell loss to the pathogenesis of this stage, but also in the evolution of the immune dysregulation to a more refractory state.

Further studies are warranted in the treatment of RRMS with estriol. Treatment of other putative Th1-mediated autoimmune diseases should also be considered, since it is likely that estriol acts through anti-inflammatory mechanisms that may or may not involve additional neuroprotective effects. A neuroprotective role for estriol treatment remains speculative, requiring different MRI outcome measures and possibly longer treatment durations. On the other hand, an anti-inflammatory mechanism of action is supported not only by our findings, but also by the fact that other human autoimmune diseases that do not involve the CNS are improved during pregnancy when estrogen levels are increased. These include RA, uveitis, autoimmune thyroiditis, and psoriasis. Also, other experimental autoimmune disease models that do not involve the CNS are improved with estrogen treatment. These include collagen-induced arthritis, uveitis, and thyroiditis. Since estrogens can be administered orally, they would be a highly desirable treatment alternative. This may not be straightforward, however, since a retrospective study found that oral contraceptive use did not protect women from developing a first episode of MS (60). Also, in a prospective trial of 132 female patients with RA, oral contraceptive use did not significantly influence outcome in long term RA; however, there was a trend for patients with long term use to have less radiographic joint damage and a better functional level (61). Further, in a randomized, placebo-controlled trial in postmenopausal RA, hormone replacement therapy had no overall effect. However, 41.6% of the patients failed to achieve serum estradiol levels $>$100 pmol/liter and were considered poor compliers. In the remaining 58.4%, the compliers, there were significant improvements after 6 mo in articular index and visual analog pain scale compared with placebo results as well as reductions in erythrocyte sedimentation rate and early morning stiffness. This outcome suggested a potential beneficial effect in RA if sufficient estrogen levels were achieved (62).

Ultimately, the estrogen dose will need to be optimized for anti-inflammatory efficacy, then weighed against the known toxicities. Hormone replacement therapy is now considered to be of more risk than benefit in healthy menopausal women. While the risk/benefit ratio of estrogen treatment may be slightly of more risk

than benefit in preventative medicine strategies in healthy menopausal women, in whom only minimum to no toxicity is tolerable, the risk/benefit ratio in patients with a chronic autoimmune disease is quite different, with modest toxicity tolerable. The use of pregnancy doses of estriol, the safest of the three estrogens (63–65), may provide an opportunity to optimize anti-inflammatory efficacy and minimize toxicity.

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