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Androgens Alter the Cytokine Profile and Reduce Encephalitogenicity of Myelin- Reactive T Cells

Bruce F. Bebo, Jr., Jeanette C. Schuster, Arthur A. Vandenbark, and Halina Offner

Adoptive transfer of proteolipid protein 139–151-specific T cell lines was used to examine the role of androgens in regulating T cell cytokine secretion and the severity of experimental autoimmune encephalomyelitis (EAE) in the SJL mouse. In this study, we found that T cells from female mice transferred more severe EAE than T cells from male mice and that gender differences in clinical disease were due, at least in part, to differences in donor T cell cytokine secretion. T cell lines were selected from proteolipid protein 139–151-immunized female SJL mice in the presence or absence of exogenous androgens. Androgen-selected T cell lines secreted less IFN-γ and more IL-10 than untreated cell lines. Clinical disease induced by the adoptive transfer of androgen-selected T cell lines was less severe than disease induced with untreated T cell lines. Furthermore, androgen treatment of naive TCR transgenic T cells, during their first encounter with Ag, resulted in a shift in the balance of Th1/Th2 cytokines. This phenotype was maintained during subsequent stimulations in the absence of androgen. These results suggest that androgen present in the lymphoid microenvironment during the induction of an immune response can alter the development of effector T cells and may play an important role in governing gender differences in the immune response and susceptibility to autoimmune disorders. The Journal of Immunology, 1999, 162: 35–40.

Immune reactivity is greater in females than in males (1, 2). The heightened immune response in females may explain their increased incidence of autoimmune disease. Multiple sclerosis (MS) is a chronic autoimmune demyelinating disease of the central nervous system. The clinical signs of MS usually appear in young adulthood, and women with the disease outnumber men 2:1 (3). Although the mechanisms for gender dimorphism in susceptibility to MS are unclear, increasing evidence suggests that sex hormones play an important role. The first clinical signs of MS typically appear after sexual maturity (4). Increased levels of sex hormones produced during pregnancy have been reported to reduce the severity of MS (4, 5), and clinical symptoms of MS often exacerbate postpartum, a time marked by reduced sex hormone levels (4, 5). The mechanisms by which sex hormones alter the immune system and susceptibility to autoimmunity remain unknown.

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system that is induced by immunizing susceptible animal strains with myelin proteins or peptides (6). EAE is a useful model that has provided considerable insights into the pathogenesis of MS. The inflammatory CD4⁺ T cells that mediate EAE secrete Th1 cytokines, including IL-2, IFN-γ, lymphotoxins, and TNF (7–9). In contrast, Th2 cells secrete IL-4, IL-5, IL-6, IL-10, IL-13, and TGF-β, and are thought to down-regulate EAE (10, 11). Skewing of the response toward Th1 or Th2 phenotype can be influenced by the quantity of Ag, the site of immunization, the initial APCs that interact with naive T cells, and cytokines (12, 13). Attention has focused primarily on the cytokines produced by either the APC or lymphocytes. However, the role of steroid sex hormones in controlling patterns of T cell lymphokines has been the focus of several recent studies. Piccini et al. demonstrated that progesterone can favor the development of Th2 cells, and induces IL-4 mRNA in established Th1 cell lines (14). Estradiol was shown to enhance IL-10 IFN-γ and regulate TNF secretion in a dose-dependent manner (15). In addition, androgens have been shown to regulate cytokine secretion (16). Treatment of experimental animals with androgen or estrogen has been shown to alter Th differentiation and the clinical course of EAE (17–19). The influence of sex hormones on the immune response is further supported by evidence demonstrating sex hormone receptors in immunocompetent cells (20, 21).

Although an immunomodulatory function for sex hormones is supported by experimental and clinical observations, the cellular targets of these hormones in the immune system have not been clearly defined. The results of this study demonstrate for the first time that androgens can regulate cytokine synthesis of ephelitogenic T cells in vitro and alter the clinical course of adoptively transferred EAE. These data are consistent with the hypothesis that gender differences in autoimmunity can be due to the direct actions of sex hormones on immunocompetent cells during the induction of the immune response.

Materials and Methods

Animals

Age-matched (5-wk) male and female SJL/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Young adult (≤8-wk-old) SJL mice were used for all of the experiments mentioned in this study. Transgenic mice bearing the functionally rearranged BV8S2 TCR gene on a B10.PL background were kindly provided by Dr. Joan Goverman (University of Washington, Seattle). The animals were bred and housed in the Animal Resource Facility at the Portland Veterans Affairs Medical Center in accordance with institutional guidelines.

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Antigens

Mouse proteolipid protein (PLP) 139–151 (HCLGKWGLHPDF) and N-acetylated myelin basic protein 1–11 (Ac-ASQKRPSQRSK) were synthesized using solid-phase chemistry on a Synergy 432A peptide synthesizer (Applied Biosystems, Foster City, CA), and purified before use.

Adoptive transfer of EAE with T cell lines

T cell lines were developed as previously described (22). Briefly, draining lymph node and spleen cells were recovered from the animals 10 days after immunization with PLP 139–151 and CFA. The T cells were selected by stimulation for 72 h with peptide, then allowed to expand in growth media containing IL-2 (25 U/ml) for 7–10 days. The specificity of the T cell lines was routinely monitored by proliferation assay, as described previously (22). The T cells were subsequently restimulated with autologous, irradiated APC and PLP 139–151 for 72 h before adoptive transfer. A quantity amounting to 5 × 10^6 T cell blasts was transferred i.p. into naive male or female SJL mice at either the first or second restimulation. The animals were monitored daily, for clinical signs as described in Ref. 22.

Cytokine detection by ELISA

PLP 139–151-specific T cell lines were suspended to 0.5 × 10^6 cells/ml and cocultured with 5 × 10^5 irradiated spleen cells as APC in stimulation media. Cell culture supernatants were recovered at 72 h and frozen at −70°C until needed for cytokine assay. Measurement of cytokines was performed by ELISA developed in our laboratory using cytokine-specific capture and detection Abs (PharMingen, San Diego, CA). Standard curves for each assay were generated using recombinant mouse cytokines (PharMingen), and the concentration of cytokines in the cell supernatants was determined by extrapolation from the appropriate standard curve. IFN-γ and TNF-α were chosen as representative Th1 cytokines, while IL-5 and IL-10 were measured as representative Th2 cytokines since the levels of IL-4 were low and irreproducible.

Hormone treatment and adoptive transfer

Spleen cells were harvested from male and female SJL mice 10 days after immunization with PLP 139–151 and stimulated as described above in the presence or absence of 10 nM testosterone. The cells were allowed to expand for 7 days in growth media containing 25 U/ml of IL-2, before restimulation with and without 10 nM testosterone. An aliquot of cells was used to measure Ag-specific proliferation, and an additional aliquot was used to analyze cytokine secretion. The remainder of the activated T cells were transferred into naive male and female SJL hosts (5 × 10^6 cells/mouse), and the animals were monitored daily for clinical score.

Androgen and estrogen receptor mRNA detection

Splenocytes were obtained from naive male and female SJL mice, then stained with FITC-labeled anti-CD4 or Mac-1-specific Abs (PharMingen), followed by an anti-FITC, paramagnetic microbead-labeled secondary Ab. The cells were then sorted on a Macs magnetic separation column (Miltenyi Biotec, Auburn, CA). Total cellular RNA was isolated from 5 × 10^3 CD4+ (95% pure) and Mac-1+ (96% pure) cells using a modified guanidinium thiocyanate-phenol-chloroform single extraction method (23). First strand cDNA was synthesized from total RNA using MLV reverse transcriptase (BRL, Bethesda, MD). Cell equivalent amounts of cDNA (approximately 10,000 cells) were used per PCR. Each sample was normalized by PCR amplification with control primers specific for mouse actin. The normalized amount of cDNA was added for each sample, then PCR amplified with specific oligonucleotide primers specific for androgen receptor (sense, 5'-TCT CAA GAG TTT GGA TGG CTC C-3'; antisense, 5'-GAG ATG ACT TCT GCC AGC ATT TC-3'); estrogen receptor (sense, 5'-GAG ACT GTG TAA CGA GAA-3'; antisense, 5'-CAA GCC AGC AGG CTT ATT C-3'); and actin (sense, 5'-ATC TAC GAG GGC TAT GCC CTC C-3'; antisense, 5'-AAT CCT CTT CAT CCT GTC AGC-3').

Statistical analysis

Mean cytokine levels were calculated from triplicate measurements and compared using the Student’s t test. The incidence and mortality of disease were compared by χ² analysis using the Yates correction (24).

Results

Gender differences in the adoptive transfer of EAE

The SJL mouse strain is highly susceptible to the adoptive transfer of EAE and has an MHC background (H-2b) previously shown to lack antisyngeneic HY responses (25). Furthermore, the feasibility of intergender adoptive transfer for the study of gender differences in EAE has recently been reported (26). Our initial experiments were designed to verify differences in the ability of male and female T cells to transfer EAE. T cell lines were derived from PLP

![Figure 1](image-url)
139–151-immunized male and female mice and transferred into naive female recipients. The specificity of the T cell lines was confirmed before the adoptive transfer of $5 \times 10^6$ T cell blasts. No significant differences in the proliferation of male and female T cell lines were measured (data not shown). Nevertheless, animals receiving female T cell lines had a higher incidence of EAE, an increased rate of mortality, and significantly more severe clinical symptoms than animals receiving male T cell lines (Fig. 1, A and B).

Gender differences in T cell cytokine secretion

Both male and female T cell lines were predominantly CD4+, and no significant differences in the expression of adhesion molecules (L-selectin, VLA-4, LFA-1), or activation markers (OX40, CD69) were observed (data not shown). The possibility that gender differences in T cell cytokine secretion were responsible for differences in the clinical disease course was tested by measuring cytokine levels in male and female T cell lines. Spleen cells were removed from PLP 139–151-immunized male and female SJL mice and stimulated in vitro for 72 h. The cells were then placed in IL-2-containing growth media for 5–7 days before being restimulated. Cytokine secretion by male and female T cell lines after restimulation with PLP 139–151 in vitro was measured, and the results are presented in Fig. 2. Although there were no differences in Ag-specific proliferation between males and females (data not shown), significant gender differences in cytokine production were observed. Female T cell lines secreted more IFN-γ than IL-10, indicative of a Th1 response, while male T cell lines made more IL-10 than IFN-γ, indicative of a Th2 response (Fig. 2). Gender differences in cytokine secretion may be responsible for differences in the severity of adoptively transferred EAE.

Androgen modulation of encephalitogenic T cells

We tested the hypothesis that sex hormones alter T cell function by examining the effects of androgens on T cell proliferation, cytokine secretion, and the ability of activated cells to transfer EAE. T cell lines from PLP 139–151-immunized female SJL mice were selected by in vitro stimulation with or without the androgen dihydrotestosterone (DHT). Although no differences in the proliferation of DHT-treated and control cell lines were observed (data not shown), T cell lines treated with physiologic levels of DHT had a significantly lower IFN-γ to IL-10 ratio (Fig. 3A). The decreased IFN-γ:IL-10 ratio was due both to a reduction in IFN-γ and an increase in IL-10 secretion by DHT-treated cells (Fig. 3B). PLP-specific T cell lines derived from female SJL mice were also selected in the presence of testosterone. Similar to what was observed with DHT, physiologic levels of testosterone (10 nM) reduced IFN-γ, and enhanced IL-10 secretion (Fig. 4B), resulting in a significant reduction in the IFN-γ:IL-10 ratio (Fig. 4A).

Our data demonstrate that androgens can regulate the production of cytokines by mature lymphocytes. The effects of androgens on Th cell differentiation were examined by using female Vβ8.2 TCR transgenic mice. Naive Vβ8.2 transgenic mice have a high frequency of myelin basic protein Ac1–11 precursor T cells, and proliferation of naive lymphocytes to Ac1–11 can be measured in vitro (27). Cytokine levels were measured from female T cell lines

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**FIGURE 2.** Cytokine secretion by male and female PLP 139–151-specific T cell lines. T cell culture supernatants were collected 72 h after in vitro stimulation with PLP 139–151. ELISA analysis was performed in triplicate from each sample. The mean and SDs were calculated from two separate experiments. *, Differences in IFN-γ and IL-10 secretion between male and female T cell lines were significant, $p < 0.001$.

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**FIGURE 3.** DHT alters T cell cytokine secretion. PLP 139–151-specific T cell lines derived from female SJL mice were selected in the presence or absence of the androgen DHT. Cell culture supernatants were harvested 72 h after in vitro stimulation and analyzed for IFN-γ and IL-10 by ELISA. A, DHT significantly alters the IFN-γ:IL-10 ratio; B, IFN-γ is reduced and IL-10 is enhanced by treatment with DHT. *, Differences in the IFN-γ:IL-10 ratio were significant, $p < 0.001$. † Mean ± SD. ‡ Differences between untreated and DHT-treated groups.

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<table>
<thead>
<tr>
<th>T cell lines</th>
<th>Treatment</th>
<th>IFN-γ (ng/ml)</th>
<th>IL-10 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Untreated</td>
<td>81.2 ± 3.1†</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>DHT 100 nM</td>
<td>53.9 ± 1.6</td>
<td>0.98 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>10 nM</td>
<td>51.5 ± 3.3</td>
<td>0.94 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>1 nM</td>
<td>45.4 ± 3.3</td>
<td>1.10 ± 0.05</td>
</tr>
<tr>
<td>P value†</td>
<td></td>
<td>&lt; 0.0001</td>
<td>&lt;0.06</td>
</tr>
</tbody>
</table>

---
and selected in vitro with or without testosterone. Testosterone-selected T cells (testosterone treated during the first in vitro stimulation only) made significantly less IFN-γ, and had elevated levels of IL-5 and IL-10 (Table I). Testosterone treatment of either control T cells (not previously treated with testosterone) or testosterone-selected T cells significantly inhibited IFN-γ secretion, but had no effect on TNF-α, IL-5, or IL-10 production (Table I).

Since androgen treatment was shown to regulate the balance of Th1 and Th2 cytokines, we hypothesized that treatment of encephalitogenic T cell lines with androgens would reduce the severity of EAE. Female PLP 139–151-specific T cells were treated with testosterone during their in vitro stimulation before transfer into naive female SJL mice, and disease severity was compared with animals receiving untreated cell lines derived from the same donor animals, after in vitro stimulation without testosterone treatment. Animals receiving testosterone-treated cell lines had a lower incidence of disease, a decreased frequency of mortality, and less severe clinical symptoms than animals injected with untreated cells (Table II).

Estrogen and androgen receptor mRNA in purified T cells and macrophages

Our data are consistent with the notion that Th cells are sensitive to testosterone-mediated regulation. It is, however, not clear that Th cells express sex hormone receptors. To elucidate this issue, we purified CD4+ T cells and Mac-1+ macrophages from the spleens of normal SJL mice and examined these cells for the expression of sex hormone receptor mRNA. Androgen and estrogen receptor mRNA was detected in both CD4+ T cells and Mac-1+ macrophages (Fig. 5). These results indicate that both CD4+ and Mac-1+ cells can be targets for the actions of sex hormones.

Discussion

In this study, we demonstrate for the first time that gonadal hormones can directly regulate encephalitogenic T cell function and alter the clinical course of adoptively transferred EAE. EAE induced by the adoptive transfer of androgen-selected T cell lines

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**Table I. Testosterone treatment alters cytokine production by VB8.2 transgenic T cell lines**

<table>
<thead>
<tr>
<th>T cell lines</th>
<th>Treatment</th>
<th>IFN-γ (ng/ml)</th>
<th>TNF-α (ng/ml)</th>
<th>IL-5 (ng/ml)</th>
<th>IL-10 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control**</td>
<td>Untreated</td>
<td>36.8 ± 0.35</td>
<td>0.04 ± 0.006</td>
<td>0.52 ± 0.02</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>25.3 ± 0.60†</td>
<td>0.04 ± 0.008</td>
<td>0.40 ± 0.09</td>
<td>0.63 ± 0.03</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Untreated</td>
<td>23.2 ± 0.67‡</td>
<td>0.05 ± 0.005</td>
<td>1.2 ± 0.02‡</td>
<td>0.86 ± 0.06‡</td>
</tr>
<tr>
<td>Selected</td>
<td>Testosterone</td>
<td>18.8 ± 0.69‡</td>
<td>0.05 ± 0.005</td>
<td>1.0 ± 0.06‡</td>
<td>0.75 ± 0.04‡</td>
</tr>
</tbody>
</table>

* Cytokines in ng/ml.

** Spleen cells were removed from naive female Vβ8.2 TCR transgenic mice and stimulated with MBP Ac1-11 peptide with or without 10 nM of testosterone for 72 hr. The cells were expanded in IL-2 containing growth medium for 7–10 days before restimulation with testosterone or without added testosterone.

† Significant difference between untreated and testosterone-treated T cell lines (previously selected without testosterone), p < 0.0001.

‡ Significant difference between control and testosterone selected T cell lines, p < 0.0001.

§ Significant difference between control and testosterone selected T cell lines, p < 0.001.

†† Significant difference between untreated and testosterone-treated T cell lines (previously selected in the presence of testosterone), p = 0.001.

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**FIGURE 4. Testosterone alters T cell cytokine secretion.** PLP 139–151-specific T cell lines derived from female SJL mice were selected in the presence or absence of the androgen testosterone. Cell culture supernatants were harvested 72 h after in vitro stimulation and analyzed for IFN-γ and IL-10 by ELISA. A, Testosterone significantly alters the IFN-γ:IL-10 ratio; B, IFN-γ is reduced and IL-10 is enhanced by testosterone treatment. * Differences in the IFN-γ:IL-10 ratio were significant, p < 0.001. † Mean ± SD. ‡ Differences between untreated and testosterone-treated groups.
was less severe than disease induced by the transfer of control T cell lines. The reduction in clinical disease was most likely due to the effects of androgens on T cell cytokine secretion since androgen-treated T cell lines made significantly less Th1 (IFN-γ), and more Th2 (IL-10) cytokines. Furthermore, androgen treatment of naive TCR transgenic T cells during their first encounter with Ag was shown to significantly alter the balance of Th1/Th2 cytokines, and this phenotype was maintained during subsequent stimulations in the absence of added hormones. These findings indicate that the transition from naive to memory/effector T cells is a critical time for the development of gender differences in the T cell response to Ags. The effects of androgens on immunocompetent cell function are further supported in this report by the detection of androgen receptor mRNA in CD4+ T cells and Mac-1+ macrophages. These results suggest that androgens can directly alter the development of effector T cells and may play an important role in governing gender differences in the immune response and susceptibility to autoimmune disease.

Gender differences in EAE

Gender differences in murine EAE parallel gender differences known to occur in the human disease MS (26, 28, 29). Although the mechanisms for increased susceptibility of disease in females are poorly understood, findings from recent studies suggest that differential induction of CD4+ T cell subsets may be involved (19, 29). Consistent with these reports, we found that immunization of male SJL mice with PLP 139–151 induced Ag-specific T cells that secreted high levels of IL-10. In contrast, immunization of age-matched female mice induced T cells that secreted high levels of IFN-γ. Subsequent transfer of female PLP 139–151-specific T cell lines induced severe EAE symptoms, while transfer of male T cell lines induced only mild disease. The diminished severity of EAE caused by male T cells was most likely due, at least in part, to their increased production of IL-10. Not only has IL-10 been shown to inhibit inflammatory Th1 responses (13, 30), protection from EAE has been induced by treatment with IL-10 (31), and anti-IL-10 Ab therapy has been shown to exacerbate EAE (32). Our data are the first to show a sex-dependent polarization in the T cell response following immunization with an encephalitogenic peptide.

Regulation of immune cell function by sex hormones

A role for gonadal hormones in the immunoregulation of encephalomyelitis is supported by recent observations in the EAE model. Pregnancy has been shown to protect animals from EAE (33, 34), and estrogen administered at levels equal to those found during pregnancy can suppress EAE (17, 18). Castration of male animals increases the severity of EAE (35), and androgens have been shown to reduce the incidence and severity of disease (19). Androgens and estrogens are clearly involved in immunoregulation. Sex hormones have been shown to inhibit delayed-type hypersensitivity reactions (36), suppress Ag- and mitogen-induced T cell proliferation (37, 38), and alter general patterns of cytokine secretion (39). In the present study, we established that sex hormones can have direct effects on T cell cytokine synthesis and the severity of adoptively transferred EAE. Physiologic concentrations of androgens (for male mice) were found to inhibit IFN-γ and enhance IL-10 secretion by PLP 139–151-specific CD4+ T cells. Adoptive transfer of female T cell lines selected in the presence of androgen induced less severe EAE than untreated female T cell lines, a response that could be attributed to a decrease in proinflammatory cytokine synthesis. Changes in cytokine synthesis were more robust when the cells were treated during their first in vitro stimulation (data not shown), suggesting that immature T cells are more sensitive to the influence of androgens. To investigate this point further, we examined the effects of androgen treatment on naive TCR transgenic T cells. Androgen treatment of naive T cells during their first encounter with Ag caused a skewing toward Th2 cytokines, and this shift was maintained in the absence of hormone during subsequent stimulations. These findings support the concept that gender differences in EAE are formed, at least in part, by sex hormones in the lymphoid microenvironment during the development of the encephalitogenic T cell response.

Mechanisms of sex hormone actions

The mechanisms by which gonadal hormones are able to regulate immune cell function remain unclear. We have shown that androgens can regulate the expression of selected gene products since testosterone and DHT alter the synthesis of specific cytokines without affecting the expression of gene products associated with cell survival or proliferation. This idea finds support in recent studies showing that estrogen can augment the activity of the IFN-γ promoter. Fox et al. (40) found two or more functional estrogen response elements lying 0.5–3.2 kb upstream from the start of the IFN-γ transcript. Although estrogen or androgen response elements have yet to be discovered in other cytokine gene complexes, it seems likely that they will.

The direct influence of sex hormones on immunocompetent cell function is further supported by evidence demonstrating sex hormone receptors in Ag-responsive cell types. Sex hormone receptors have been detected in splenocytes, thymocytes, and peripheral blood cells.
Androgens modulate encephalitogenic T cells.


