Evidence That Androgens Modulate Human Thymic T Cell Output

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Background: The thymus has long been recognized as a target for the actions of androgenic hormones, but it has only been recently recognized that alterations in circulating levels of gonadal steroids might affect thymic output of T cells. We had the opportunity to examine parameters of thymic cellular output in several hypogonadal men undergoing androgen replacement therapy.

Methods: Circulating naive ($CD4^+CD45RA^+$) T cells were quantitated by flow cytometric analysis of peripheral blood mononuclear cells. Cells bearing T-cell receptor excision circles were quantitated using real-time polymerase chain reaction amplification of DNA isolated from peripheral blood mononuclear cells from healthy men and from hypogonadal men before and after testosterone replacement therapy.

Results: $CD4^+CD45^+$ (naive) T cells comprised 10.5% of lymphocytes in healthy males; this proportion was greatly increased in 2 hypogonadal men (35.5% and 44.4%). One man was studied sequentially during treatment with physiologic doses of testosterone. $CD4^+CD45RA^+$ cells fell from 37.36% to 20.05% after 1 month and to 12.51% after 7 months of normalized androgen levels. In 2 hypogonadal patients, T-cell receptor excision circle levels fell by 83% and 78% after androgen replacement therapy.

Conclusions: Our observations indicate that the hypogonadal state is associated with increased thymic output of T cells and that this increase in recent thymic emigrants in peripheral blood is reversed by androgen replacement.

Key Words: androgens, thymus, recent thymic emigrants, T cells

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It has been recognized for more than a century that androgen deprivation in adult male animals is associated with thymic enlargement.[1] More recent studies have revealed that androgen deprivation results in re-expansion of the involuted thymus of adult animals[2] with consequent changes in the complement of circulating T cells.[3–5] These effects of gonadal steroids on T-cell development are being actively explored as possible approaches to a variety of clinical problems ranging from correction of declining immune system function that accompanies normal aging,[6] to immune reconstitution scenarios after immune ablation by cytotoxic therapy,[7,8] to amelioration of autoimmune disease.[9]

Studies of the thymus in humans have historically been more difficult because the organ is not readily sampled in vivo. However, recently developed tools are now available, which permit assessment of thymic function in humans in health and disease states. T cells that have recently left the thymus for the periphery may be detected using flow cytometric detection of specific surface markers. $CD4^+$ T lymphocytes that are exported from the thymus express the surface antigen CD45RA, and the number of cells coexpressing these 2 markers was recognized to correlate with thymic expansion observed during recovery from cytotoxic chemotherapy[10] or during immune system recovery during antiviral treatment of human immunodeficiency virus.[11] A second method for the assessment of thymic cellular output is the detection of T-cell receptor excision circles (TRECs) that are present in recent thymic emigrants. These episomes are a result of the normal process of T-cell receptor rearrangement that occurs during thymocyte maturation.[12,13] Because the TREC sequences do not replicate with the cell, they are present in only those cells that have recently emigrated from the thymus.

We applied these techniques to address whether evidence of increased thymic output of T cells could be observed in men with acquired hypogonadism and whether androgen replacement therapy was associated with a reversal of that increased thymic output.

MATERIALS AND METHODS

Patients and Control Subjects

Six male patients with hypogonadism were studied (Table 1). The age range was from 33 to 64 years. Baseline free testosterone levels ranged from 17.4 to 45.5 pg/mL (normal range, 47.0–244.0 pg/mL). All patients were evaluated by an endocrinologist to determine causes of hypogonadism. Gonadal failure was primary in 3 patients. Two patients had hypogonadotrophic hypogonadism, and both of these also had type 2 diabetes mellitus. One subject had a prolactinoma, and medical treatment of the tumor was associated with return of testosterone levels to the normal range. Two subjects were studied at baseline and after normalization of serum testosterone. The healthy control group included 5 healthy males ranging in age from 26 to 70 years. These studies were approved by the Committee for the Protection of Human Subjects of the Institutional Review Board.

Preparation of Blood Samples

A heparinized blood sample (25 mL) was obtained and a small volume reserved to determine total white blood cell count and differential. The remainder was used to prepare peripheral blood mononuclear cells by centrifugation at room temperature on Ficoll gradients (density, 1.077; Sigma Chemical Co, St Louis, MO). Mononuclear cells were removed from the interface of the gradients, washed and used for flow cytometry or to prepare genomic DNA.

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TABLE 1. Hypogonadal Subjects and Studies Performed

Subject No.	Age, yr	Diagnosis	Free Testosterone, pg/mL*	Studies Performed		
				TRECs	Naive T Cells	Testosterone Replacement
1	56	Primary hypogonadism	44.9		✓	
2	53	Hypogonadotrophic hypogonadism	43.1		✓	
3	56	Hypogonadotrophic hypogonadism	21.0		✓	
4	33	Hyogonadism due to hyperprolactinemia	17.4		✓	
5	42	Primary hypogonadism	43.0	✓	✓	✓
6	64	Primary hypogonadism	45.5	✓		✓

T-cell receptor excision circles quantitated by real-time PCR.
Naive T cells: $CD45RA^+$ cells quantitated by flow cytometry.
Testosterone replacement: studies repeated after androgen repletion.
*Normal range, 47.0–244.0 pg/mL.

Flow Cytometry

Peripheral blood mononuclear cells were suspended at 1×10^6/mL in phosphate buffered saline with 2% bovine serum albumin and azide. Conjugated monoclonal antibodies were added to cells according to the manufacturer's directions. Cells were stained with the following combinations of antibodies: CD4 + CD8 and CD45RA/CD45RO/CD3/CD4 (Simultest and Multitest, respectively; Becton Dickinson, San Jose, CA). Stained cells were incubated at 4°C for 30 minutes, washed, and analyzed with a FACStar Plus (Becton Dickinson Immunocytometry Systems).

T-Cell Receptor Excision Circle Measurement

DNA was extracted from PBMC pellets using the QIAamp DNA kit (Qiagen, Valencia, CA). T-cell receptor excision circles were measured using a Taqman real-time polymerase chain reaction (PCR) assay to amplify and quantitate PCR products for signal-joint TRECs and β-actin control sequences. Reaction mixtures (50 μL) contained 100 ng genomic DNA and the following other components: Taqman Universal PCR Master mix (2×), 300 nmol each TREC primer, and 250 nmol of each probe (see sequences given). Temperature cycling on an ABI 7300 began with 2 initial holds at 50°C for 2 minutes and 95°C for 10 minutes. These were followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Sequences were as follows:
Sense: TREC5:
5′-CATCCCTTTCAACCATGCTGACACCTCT-3′
Antisense: TREC3:
5′-CGTGAGAACGGTGAATGAAGAGCAAGACA-3′
Sense: β-actin5:
5′-TCACCCACACTGTGCCCATCTACGA-3′
Antisense: β-actin3:
5′-CAGCGGAACCGCTCATTGCCAATGG-3′

Sequences for the 2 labeled probes were as follows:
TREC:
5′-VIC-TTTTTGTAAAGGTGCCCACTCCTGTG CACGGTGA-3′
β-actin:
5′-FAM-ATGCCCTCCCCCATGCCATCCTGCGT-3′

Standardization was done using stock DNA from a young control subject that was positive for TRECs, and the same stock was included in each experiment. Standard curves for both TREC and β-actin included six 2-fold dilutions of stock DNA and were run in each experiment. The standards showed a linear relationship between the cycle at which fluorescence was first significantly detected above background (threshold cycle; C_T) and the log concentration of DNA (ng; $R^2 > 0.98$). DNA samples from each patient and control subject were performed in duplicate, and mean values were used for conversion to quantitative units.

RESULTS

Naive T Cells

Five of the 6 hypogonadal patients had naive T cells in peripheral blood quantitated by flow cytometry. The hypogonadal patients had lymphocyte counts in the normal range, and total numbers of circulating CD4 and CD8 T lymphocytes also were not significantly different from the control group (data not shown). Naive T cells within the CD4 population identified by coexpression of CD45RA represented a mean ± SEM of 10.55% ± 1.87% of the healthy male lymphocytes. Hypogonadal males were found to have 20.49% ± 7.99% naive T cells; this was not statistically different from the finding in healthy males in this small sample. The oldest healthy subject (aged 70 years) had the lowest percentage of naive T cells (3.6%). Two hypogonadal patients (subjects 4 and 5) had the highest levels of this naive T-cell population, with values of 42% and 37% (Fig. 1A). Subject 4 had the lowest serum testosterone level in the group (17.4 pg/mL) and the highest proportion of $CD4^+CD45RA^+$ cells; however, testosterone levels did not show a significant correlation with the naive T cell phenotype in the group as a whole (not shown). Most notable was the fact that both patients with high naive T-cell numbers were less than 45 years of age, whereas hypogonadal patients older than 50 years had naive T-cell numbers comparable to healthy controls of similar age. The inverse correlation of naive T-cell percent with age was statistically significant ($r^2 = 0.86$; $P = 0.023$) for the hypogonadal men but not for the healthy controls ($r^2 = 0.31$; $P = 0.392$). These data suggest that age is a significant factor in the potential for expansion of naive T cells in the hypogonadal state.

Flow cytometry data for 2 hypogonadal men with elevated numbers of naive T cells are shown in Figure 1B. Although the 2 healthy men (right panels) show only a small fraction of $CD4^+$ cells staining with CD45 (upper right quadrant of each panel), this population is greatly expanded in the samples from the 2 hypogonadal men.

T-Cell Receptor Excision Circles

We sought to corroborate the findings of increased naive T-cell numbers by assessing whether recent thymic emigrant cells containing TRECs were also increased in hypogonadal

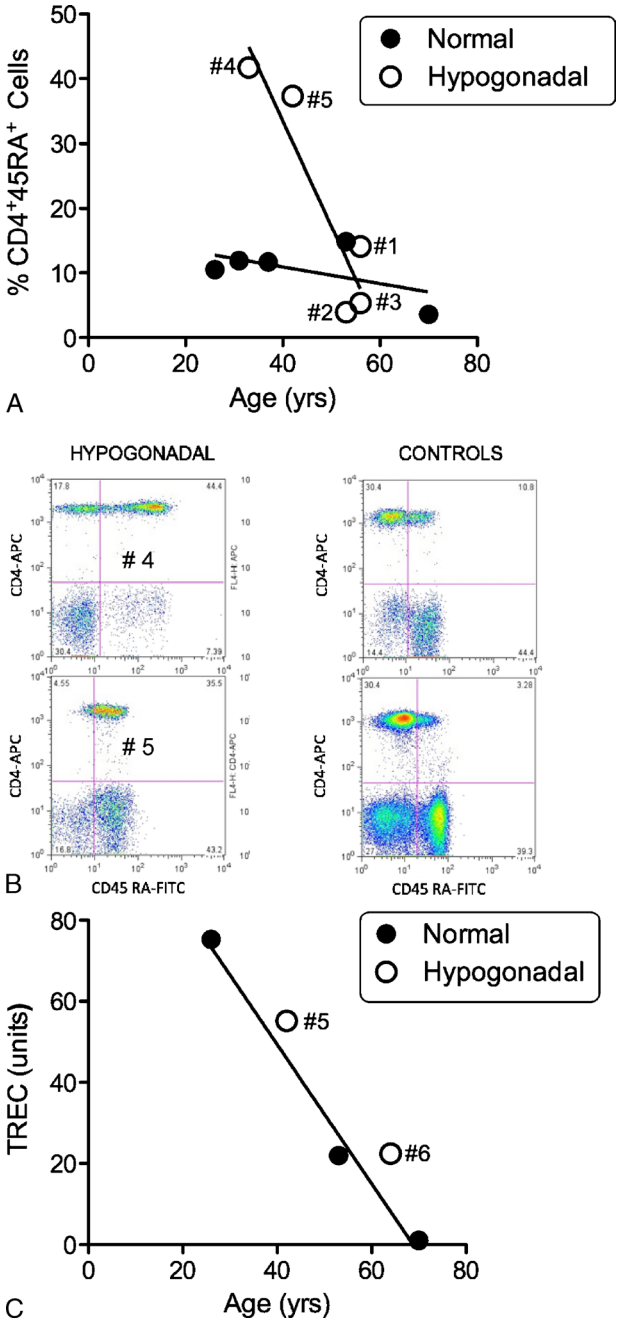

FIGURE 1. A, $CD4^+CD45RA^+$ T cells (percent of total cells) as a function of age in 5 healthy males (closed symbols) and in 5 hypogonadal males (open symbols with subject numbers from Table 1). B, Flow cytometric data showing staining for CD4 and CD45RA in 2 hypogonadal males (left panels; with subjects 4 and 5 from Table 1) and 2 healthy control men (right panels). $CD4^+CD45RA^+$ cells are in the right upper quadrant of each panel. C, T-cell receptor excision circle quantitation as a function of age in 3 healthy control males (closed symbols) and in 2 hypogonadal men (open symbols with subject numbers from Table 1).

men. Figure 1C shows the relative abundance of TRECs measured by Taqman assay plotted as a function of age in 3 healthy men and 2 hypogonadal men. With this small sample, no difference could be assessed between healthy and hypogonadal men.

Androgen Effects on $CD4^+CD45RA^+$ Cells and on TRECs

In several instances, we had the opportunity to examine parameters of thymic T-cell output during androgen replacement. One hypogonadal man (subject 5) had flow cytometry studies performed on 3 separate visits. The first sample was collected before initiation of testosterone treatment, and subsequent samples were collected after 1 month and 7 months of therapy. His initial level of free testosterone (43.0 pg/mL) was increased into the normal range (123.7 pg/mL) after treatment with topical testosterone gel. The CD4:CD8 ratio was decreased from 1.26 at baseline to 1.12 after treatment. Numbers of $CD45RA^+$ naive T cells were elevated in both the CD3 and CD4 compartments before treatment. $CD4^+CD45^+$ cells were decreased to around 50% of baseline levels after 1 month of testosterone therapy, and this was maintained during an additional 6 months of treatment (Fig. 2A).

Two individuals (subjects 5 and 6) had measurements of TRECs performed in the hypogonadal state and during testosterone therapy (Fig. 2B). Each showed a remarkable reduction in TREC numbers with hormonal replacement therapy to restore physiologic androgen levels. Subject 5's TREC levels fell by 78%; TRECs in subject 6 fell by 87% after androgen replacement.

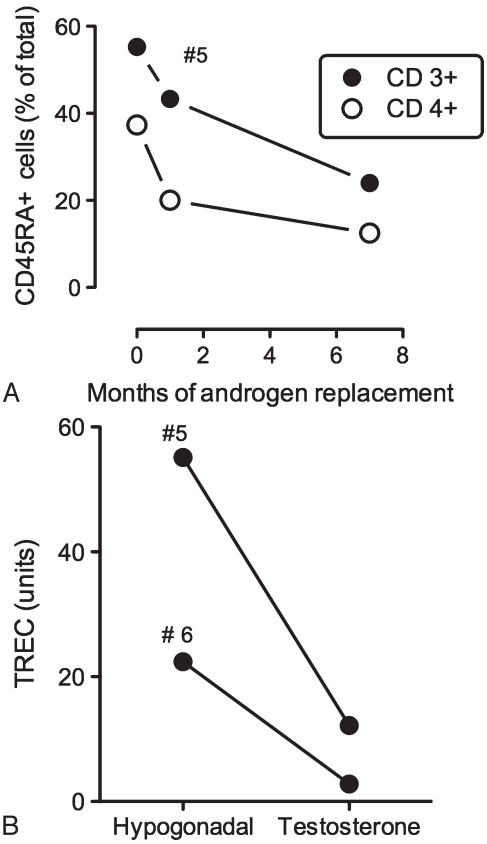

FIGURE 2. A, $CD45RA^+$ cells (as percent of total) during androgen replacement therapy of subject 5 (Table 1) with hypogonadism. $CD3^+$ cells are shown in closed symbols, and $CD4^+$ cells are shown in open symbols. B, T-cell receptor excision circle quantitation in two men (subjects 5 and 6 from Table 1) in the hypogonadal state and after restoration of normal testosterone levels.

DISCUSSION

The data presented here are some of the first to show evidence in humans of an effect of androgenic hormones on parameters of thymic function. In humans, treatment with luteinizing hormone–releasing hormone analogs, with resultant hypogonadotropic hypogonadism, has been found to result in increases in total peripheral T cells, including both $CD4^+$ and $CD8^+$ cells, as well as increases in recent thymic emigrants marked by CD45RA expression or by the presence of TRECs.[5] Our current report confirms the finding of increased numbers of naive T cells in the circulation of two androgen-deficient men. The hypogonadal states of our subjects were not pharmacologically induced, and we included both men with primary testicular failure and men with hypogonadotropic hypogonadism due to hypothalamic or pituitary disease. Furthermore, we demonstrate evidence for the first time in humans that restoration of physiologic levels of testosterone results in reversal of this increased thymic output of T cells.

The chief limitations of our study are the small numbers of subjects examined, their heterogeneous clinical features, and that we did not have the opportunity to perform both assessments (naive T cells and TRECs) in each subject. Likely because of the small numbers of subjects studied, we did not see statistically significant differences between healthy and hypogonadal groups of men with respect to either naive T cells or TRECs, whereas individual subjects showed dramatic reductions in $CD45RA^+$ cells and in TRECs with androgen replacement. Detailed analyses of larger numbers of men with various types of spontaneously occurring or pharmacologically induced hypogonadism will be of considerable interest.

Studies of the influence of androgens on the immune system are being actively pursued for a number of reasons. Immune reconstitution after cytotoxic treatment directed at neoplastic disease has been found to be accelerated by temporary attenuation of androgen effects.[4,7,8] This may be of considerable importance in older individuals, in whom immune function defects only slowly, if ever, resolve after cytotoxic therapy.[7] In addition, recognition of a role for androgens in immune modulation and advances in our understanding of underlying mechanisms has potential for advancing development of new approaches to attenuate a variety of autoimmune disorders.

The mechanisms by which androgens exert their effects on the thymus and, consequently, the peripheral immune system remain to be explored. Although signaling systems for these hormones are expressed in thymocytes,[14] it seems more likely that the some of the physiologic effects leading to androgen-induced thymic regression are exerted at the level of the thymic epithelial cells,[15] and studies of the effects of androgen depletion on immune reconstitution have shown an augmented proliferative response of thymic epithelial cells under such conditions of androgen deprivation.[8] In the androgen-deficient thymus, an increased rate of immigration of bone marrow T-cell precursors occurs, and the developmental niche for these cells within the gland is enhanced by proliferation of thymic medullary epithelial cells and their increased production of CCL25, an important ligand for immigration of early thymic progenitor cells.[16]

Our results reported here extend the observations of Sutherland and colleagues,[5] showing for the first time that androgen deficiency in males, arising both from either gonadotropin deficiency or from primary testicular disease, is associated with increased thymic output of cells and that this increase is reversed upon restoration of physiologic levels of androgenic hormones in men. Continued exploration of the cellular and molecular targets of androgens in the human immune system may open new therapeutic avenues in clinical settings as diverse as immune reconstitution and autoimmune disease.

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