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In Vivo Effects of Sex Steroids on Lymphocyte Responsiveness and Immunoglobulin Levels in Humans

E. J. GILTAY, J. C. M. FONK, B. M. E. VON BLOMBERG, H. A. DREXHAGE, C. SCHALKWIJK, AND L. J. G. GOOREN

Research Institute for Endocrinology, Reproduction, and Metabolism (E.J.G., L.J.G.G.), Departments of Pathology (J.C.M.F., B.M.E.B.) and Clinical Chemistry (C.S.), University Hospital Vrije Universiteit, 1007 MB Amsterdam; and Department of Immunology, Erasmus University (H.A.D.), Rotterdam, The Netherlands

ABSTRACT

The female predominance in several autoimmune diseases suggests a role for sex steroid hormones in disease susceptibility. We therefore investigated to what extent sex hormones would influence immune responsiveness. We analyzed T helper type 1 (T_H1) and type 2 cytokine patterns, chemokine receptor expression ($n = 2 \times 10$), and Ig levels ($n = 2 \times 25$) in transsexual men and women before and after 4 months of cross-sex hormone administration. Antithyroperoxidase levels were compared between 186 transsexual males (treated >5 yr with estrogens) and 186 male controls.

In men, estrogens plus antiandrogens increased free cortisol levels in 24-h urine samples, decreased natural killer cell numbers, and slightly inhibited the mitogen-induced interferon-γ/interleukin-4 ratio, but up-regulated the expression of T_H1-associated chemokine receptors, CCR1, CXCR3, and CCR5. Conversely, in women, androgens slightly decreased free cortisol levels in 24-h urine samples and enhanced the mitogen-induced interferon-γ/interleukin-4 ratio and tumor necrosis factor-α production. At the single cell level no T_H1/T_H2 shifts were found. Remarkably, up-regulation of T_H1 cytokines was accompanied by down-regulation of CCR1, CXCR3, and CCR5 expression. Neither $CD4^+$ lymphocyte numbers nor IgG, IgM, and antithyroperoxidase levels, although higher in women then in men, were affected by cross-sex hormonal treatment.

These results demonstrate that the capacity to develop a T_H1 phenotype of peripheral blood lymphocytes is stimulated by androgens and is slightly inhibited by estrogens. These changes may be direct or indirect through the effects on other hormones. (*J Clin Endocrinol Metab* **85:** 1648–1657, 2000)

SEVERAL LINES of evidence implicate sex steroids as modulators of immune responses, but the *in vivo* mechanisms underlying this relationship are poorly understood (1). A first important observation is the higher prevalence of most autoimmune diseases in females compared to males (2). Second, women compared to men, have higher serum levels of IgM, IgG (3–6), and distinct autoantibodies such as IgG against the major microsomal antigen thyroperoxidase (TPO-Ab) (7–9). Conversely, women compared to men, show a reduced antibody-dependent cell-mediated (10, 11) and natural killer (NK) cell cytotoxicity (12, 13). Third, binding sites for sex steroids are present (14–16), and sex steroids can be metabolized in immunocompetent cells (17–19), suggesting that sex steroids may affect leukocyte function directly. Fourth, experimental studies show that the course of autoimmune disease can be modulated by castration or administration of sex steroids, for example in chicken and murine models of autoimmune thyroid disease (20, 21). Estrogen treatment in female or male mice as well as castration of male mice result in an increase in autoantibody levels (21–25).

T helper cells can be divided into the reciprocally suppressive T helper type 1 (T_H1) and T_H2 subsets, which are defined by their profile of cytokine release and the type of response they elicit. Polarized T_H1 or T_H2 immune responses are associated, on the one hand, with protective immunity against certain intracellular bacteria and viruses or parasites, respectively, and, on the other hand, with certain autoimmune diseases or allergy. T_H1 cells produce interleukin-2 (IL-2), interferon-γ (IFNγ), and, less specifically, tumor necrosis factor-α (TNFα), stimulating cell-mediated immune responses, whereas T_H2 cells produce IL-4, IL-5, IL-10, and IL-13, which provide help for humoral immune responses (26, 27), such as the production of IgE and IgG4 (28, 29). The body distribution of these T cells is directed by the differential expression on the cell membrane of distinct sets of adhesion molecules and chemokine receptors. Chemokines are chemotactic cytokines produced by a wide variety of cells to attract the relevant leukocytes to sites of infection and inflammation (30, 31). T_H1 and T_H2 cells express different chemokine receptor profiles; T_H1 cells seem to preferentially express the CC chemokine receptors, CCR1, CXCR3, and CCR5 (32–35), facilitating their selective migration into inflammatory lesions. The factors that influence chemokine receptor expression *in vivo* are still largely unknown.

Sex differences on cytokine production *in vivo* have not been reported to date; nevertheless, sex steroids seem to differentially affect T_H1 and T_H2 cytokine production. During pregnancy, the T_H1/T_H2 balance is skewed toward T_H2 (36), thereby preventing rejection of the antigenically foreign fetus by a cell-mediated immune attack (37, 38). The shift toward a T_H2 response may be triggered by increased circulating levels of 17β-estradiol and progesterone (39) during pregnancy (40). The *in vitro* influence of sex steroids on T cell cytokine production has been studied extensively (17, 18, 39,

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Address all correspondence and requests for reprints to: Erik J. Giltay, M.D., Department of Endocrinology, Division of Andrology, University Hospital Vrije Universiteit, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands. E-mail: giltay@dds.nl.

41, 42), showing complex and diverse effects, but human *in vivo* studies apart from pregnancy are scarce. Healthy transsexual males and females, therefore, provide a unique opportunity to study the *in vivo* effects of cross-sex steroid hormones on the immune system. Here we examine 1) the effects of either estrogens plus antiandrogens or testosterone on normal humoral and cellular immune parameters, and 2) the incidence of TPO-Ab expression in transsexual males treated for at least 5 yr with estrogens compared to male controls.

Subjects and Methods

Subjects in prospective study

We included 30 Caucasian male to female (M→F; median age, 26 yr; range, 18–45) and 30 Caucasian female to male (F→M) transsexuals (median age, 23 yr; range, 17–40). The body mass index was 20 ± 3 kg/m^2 (mean ± SD) in M→F and 23 ± 5 kg/m^2 in F→M transsexuals. All measurements were performed before and again after 4 (and 12) months of cross-sex hormone administration. In both groups gonadectomy had not yet taken place, but after cross-sex hormone administration their own sex steroid production was suppressed (Table 1). M→F transsexuals were treated with ethinyl estradiol (100 μg/day; Lynoral, Organon, Oss, Netherlands) in combination with the antiandrogen cyproterone acetate (100 mg/day; Androcur, Schering AG, Weesp, The Netherlands). F→M transsexuals were treated with either im testosterone esters (n = 27; Sustanon, Organon; 250 mg/2 weeks) or oral testosterone undecanoate (n = 3; Andriol, Organon; 160 mg/day). One F→M transsexual reported intake of oral contraceptives 6 months before baseline. All other F→M transsexuals had had regular menstrual cycles (28–31 days) before cross-sex hormone administration. There was no evidence of autoimmune disease, immune deficiencies, chronic infection (*e.g.* with human immunodeficiency virus type 1), endocrine diseases, or use of other sex hormones for 3 yr or more before baseline. In all subjects, the body mass index (weight/height^2) was assessed, venous blood samples were taken in the morning between 0900–1200 h after an overnight fast, and 24-h urine was collected (during the 24 h before blood sampling). For logistical reasons, most measurements were obtained in randomly chosen subgroups (Table 1). The investigation conformed with the principles outlined in the Declaration of Helsinki. Informed consent was obtained from all subjects, and the study was approved by the ethical review committee of the University Hospital Vrije Universiteit.

Endocrine measurements

Standardized RIAs were used to determine serum levels of testosterone (Coat-A-Count, Diagnostic Products, Los Angeles, CA), 5α-dihydrotestosterone (after extraction, Intertech, Strassen, Luxembourg), dehydroepiandrosterone sulfate (DHEAS; Diagnostic Products), 17β-estradiol (Sorin Biomedica, Saluggia, Italy), and, in 24-h urine samples, free cortisol (after extraction; Coat-A-Count, Diagnostic Products). To assess peripheral androgen activity, we measured serum 5α-androstane-3α,17β-diol glucuronide (Adiol G) (43) by RIA (Diagnostic Systems Laboratories, Inc., Webster, TX). Immunometric luminescence assays were used to determine levels of FSH (Amerlite, Amersham Pharmacia Biotech, Aylesbury, UK) and LH (Amerlite). Immunoradiometric assays were used to measure serum levels of GH (Sorin Biomedica, Saluggia, Italy) and PRL (Biosource Technologies, Inc., Fleurus, Belgium).

Lymphocyte phenotyping

Measurements of lymphocyte subpopulations, cytokines, and chemokine receptors were performed in a random subgroup of 10 M→F transsexuals (median age, 27 yr; range, 20–41) and 10 F→M transsexuals (median age, 26 yr; range, 18–40) at baseline and after 4 months of cross-sex hormone administration. In fresh heparinized venous blood the numbers of CD3-, CD4-, CD8-, CD19-, and CD16/56-positive cells were analyzed on a FACStar Plus (Simulset, Becton Dickinson and Co., San Jose, CA). These lymphocyte subpopulations are expressed as percentages of the total number of lymphocytes.

Cytokine production in supernatants

IFNγ, IL-2, TNFα, IL-4, IL-5, and IL-10 production by Ficoll isolated (Lymfoprep, Nycomed, Pharma AS, Oslo, Norway) and cryopreserved peripheral blood mononuclear cells (PBMC) was assessed in culture supernatants. To eliminate the effect of interassay variation, pre- and posttreatment samples from the same subject were analyzed in the same run. Therefore, 2.5×10^6 PBMC/mL were cultured for 36 h in Iscove's Modified Dulbecco's Medium (BioWhittaker, Inc., Verviers, Belgium) supplemented with 10% human pooled serum (Central Laboratory Blood transfusion, Amsterdam, The Netherlands), 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2 μg/mL anti-CD25 (CLB clone TB30). Cells were stimulated with 10 μg/mL phytohemagglutinin (PHA; Murex Diagnostics Ltd., Dartfort, UK). Supernatants were stored at −80 C until analyses. All cytokines were measured by standard enzyme-linked immunosorbent assay techniques with the following commercially available antibodies or kits: IL-2 and IFNγ (Medgenix Diagnostics SA, Fleurs, Belgium); IL-4, IL-6, and TNFα (CLB compact enzyme-linked immunosorbent assay); and IL-10 (PharMingen, San Diego, CA).

Cytokines production at the single cell level

Staining of intracellular cytokines was performed as described by Jung *et al.* (44). In brief, 2×10^6/mL thawed PBMC were incubated for 4 h in Iscove's Modified Dulbecco's Medium with the following supplements: 10% FBS (Integro, Leuvenheim, The Netherlands), penicillin, streptomycin, 20 ng/mL phorbol myristate acetate (Sigma, St. Louis, MO), 1 μmol/L calcium ionophore (Sigma), and 3 μmol/L monensin (Sigma). Subsequently, the cells were washed with phosphate-buffered saline/0.5% BSA (Roche Molecular Biochemicals, Mannheim, Germany) and stained with anti-CD3-RPE-Cy5 (DAKO Corp., Glostrup, Denmark) and anti-CD8-fluorescein isothiocyanate (anti-CD8-FITC; Becton Dickinson and Co.). After washing, the cells were fixed with 4% paraformaldehyde (Merck & Co., Inc., Darmstadt, Germany) and permeabilized with phosphate-buffered saline with 0.1% saponin (Merck & Co., Inc.), 0.5% BSA, and 10% human pooled serum. Intracellular staining was performed with anti-IFNγ-phycoerythrin (anti-IFN-PE), anti-IL-4-PE, and TNFα-PE (PharMingen). Cells were analyzed on a FACStar Plus. The percentages of cytokine-producing cells were calculated within the $CD3^+$, $CD3^+/CD8^+$, and $CD3^+/CD8^-$ cell fractions.

Chemokine receptor expression at the single cell level

Unstimulated PBMC were incubated for 30 min with biotinylated anti-CCR1 (R&D Systems Europe, Abingdon, UK), anti-CXCR3-FITC (R&D Systems Europe), or anti-CCR5-FITC (PharMingen). The cells were additionally stained with anti-CD3-RPE-Cy5 (DAKO Corp.) and anti-CD8-PE (Becton Dickinson and Co.). The cells incubated with anti-CCR1 cells were subsequently stained with streptavidin-FITC (DAKO Corp.). After analyses on a FACStar Plus, the geometric mean surface expression as well as the percentages of chemokine receptor-positive cells were calculated within the $CD3^+$, $CD3^+/CD8^+$, and $CD3^+/CD8^-$ cell fractions.

Serum levels of Ig (sub)classes

Measurements were performed in 25 M→F transsexuals (median age, 29 yr; range, 18–43) and 25 F→M transsexuals (median age, 23 yr; range, 16–37) at baseline and after 4 months of cross-sex hormone administration and in a subgroup of 13 M→F and 13 F→M transsexuals again at 12 months. Serum levels of IgA, IgM, IgG, and IgG4, the human equivalent of murine IgG1, were assessed by kinetic nephelometry [Array Protein System 360, Beckman Coulter, Inc., Fullerton, CA; interassay coefficients of variation (CV), <2.5%; interassay CV, <2.8%); IgE was assessed by fluoroimmunoassay (Pharmacia CAP System, Pharmacia & Upjohn, Inc., Bridgewater, NJ; intraassay CV, 6%; interassay CV, 6%).

Cross-sectional study of TPO-Ab expression

We included 186 M→F transsexuals [median age, 43 yr; range, 21–70, because a sex difference in having TPO-Ab was found in the age range

TABLE 1. Endocrine and immune parameters at baseline and 4 months of cross-sex hormone treatment in 30 M → F and 30 F → M transsexuals

	M → F transsexuals				F → M transsexuals				P^c
	n	Baseline	4 months	P^a	n	Baseline	4 months	P^a	
Body mass index (kg/m^2)	30	21.3 ± 2.7	22.0 ± 2.5	<0.001	30	23.8 ± 4.9	24.9 ± 4.7	0.004	0.08
Hormone levels									
17β-Estradiol (pmol/L)	30	99.5 ± 19.5	[c]	[c]	30	187 ± 132	134 ± 49	0.02	<0.001
Total testosterone (nmol/L)	30	22.4 ± 6.3	1.0 ± 0.0	<0.001	30	1.8 ± 0.6	28.5 ± 13.3	<0.001	<0.001
5α-Dihydrotestosterone (nmol/L)	15	3.09 ± 1.33	0.30 ± 0.19	0.001	13	0.70 ± 0.17	2.34 ± 0.67	0.001	<0.001
Adiol G (nmol/L)	16	24.6 ± 12.3	2.8 ± 1.5	<0.001	18	15.1 ± 16.3	35.2 ± 14.1	0.001	0.001
DHEAS (μmol/L)	15	8.6 ± 2.4	5.5 ± 1.3	0.001	13	6.5 ± 2.1	6.1 ± 2.1	0.58	0.04
Free cortisol (nmol in 24-h urine)	15	156 ± 74	257 ± 148	0.008	12	113 ± 36	93 ± 41	0.12	0.16
LH (U/L)	30	2.8 ± 1.6	0.3 ± 0.1	<0.001	30	4.7 ± 3.2	2.3 ± 2.3	<0.001	0.007
FSH (U/L)	30	2.7 ± 1.8	0.5 ± 0.1	<0.001	30	4.3 ± 1.4	3.3 ± 1.7	0.04	0.001
PRL (U/L)	30	0.15 ± 0.06	0.47 ± 0.19	<0.001	30	0.25 ± 0.16	0.24 ± 0.13	0.53	0.001
GH (U/L)	15	2.9 ± 4.3	7.6 ± 8.4	0.07	13	9.5 ± 9.3	7.1 ± 8.1	0.23	0.04
Peripheral blood cells									
Leukocytes (×10^9/L)	30	7.4 ± 2.3	9.0 ± 2.4	<0.001	30	7.7 ± 2.7	8.1 ± 2.8	0.19	0.91
Lymphocytes (×10^9/L)	10	1.8 ± 0.7	1.7 ± 0.7	0.95	10	2.0 ± 1.0	2.1 ± 0.6	0.59	0.74
CD4$^+$ T cells (% of lymphocytes)	10	41.4 ± 7.5	43.2 ± 6.1	0.41	10	48.0 ± 4.5	47.5 ± 5.1	0.67	0.05
CD8$^+$ T cells (% of lymphocytes)	10	31.6 ± 6.4	32.2 ± 8.4	0.91	10	28.5 ± 5.2	28.7 ± 6.0	0.89	0.27
CD4/CD8 ratio	10	1.38 ± 0.42	1.45 ± 0.44	0.44	10	1.75 ± 0.40	1.73 ± 0.45	0.89	0.08
CD19$^+$ B cells (% of lymphocytes)	10	13.3 ± 3.1	15.6 ± 3.5	0.11	10	14.3 ± 4.0	13.8 ± 3.9	0.29	0.97
CD16$^+$ or 56$^+$/CD3$^-$ NK cells (% of lymphocytes)	10	14.9 ± 9.5	8.8 ± 3.2	0.01	10	11.0 ± 5.8	12.2 ± 8.4	0.28	0.32
Ig isotype serum levels									
IgA (g/L)	25	1.45 (1.15–1.83)	1.30 (1.03–1.64)	0.002	25	1.57 (1.35–1.82)	1.47 (1.27–1.70)	0.001	0.81
IgE (U/mL)	25	76.7 (44.4–132.6)	69.2 (41.5–115.5)	0.08	25	42.8 (23.7–77.3)	39.9 (22.3–71.4)	0.61	0.20
IgM (g/L)	25	0.92 (0.73–1.15)	0.94 (0.75–1.22)	0.81	25	1.38 (1.15–1.66)	1.48 (1.22–1.79)	0.009	0.006
IgG (g/L)	25	9.0 (8.1–10.0)	8.8 (7.9–9.7)	0.12	25	10.3 (9.5–11.1)	10.1 (9.2–11.0)	0.35	0.04
IgG4 (g/L)	25	0.54 (0.38–0.75)	0.50 (0.35–0.71)	0.10	25	0.40 (0.25–0.62)	0.40 (0.25–0.62)	0.93	0.37
IgG4/IgG ratio (%)	25	5.97 (4.41–8.06)	5.65 (4.14–7.71)	0.049	25	3.87 (2.52–5.92)	3.92 (2.58–5.98)	0.64	0.14
Intracellular cytokine expression in CD3$^+$ T cells									
IFNγ (% of positive cells)	9	17.4 (13.8–21.9)	15.4 (12.0–19.7)	0.29	9	12.6 (9.6–16.6)	13.7 (10.1–18.6)	0.68	0.07
IL-4 (% of positive cells)	9	10.7 (8.3–13.8)	11.3 (7.6–16.9)	0.67	9	13.5 (9.9–18.3)	12.1 (9.3–15.9)	0.14	0.11
TNFα (% of positive cells)	9	38.9 (32.3–46.9)	33.5 (23.6–47.5)	0.68	10	42.0 (35.1–50.3)	38.5 (32.9–45.0)	0.58	0.80
IFNγ/IL-4 ratio	9	1.62 (1.16–2.27)	1.36 (0.89–2.08)	0.77	10	1.01 (0.66–1.53)	1.10 (0.79–1.53)	0.26	0.03
CD8$^+$/IFNγ/IL-4 ratio	9	3.08 (2.13–4.45)	1.93 (0.97–3.85)	0.14	10	1.89 (1.16–3.06)	1.73 (1.24–2.41)	0.96	0.07
CD8$^-$/IFNγ/IL-4 ratio	9	1.04 (0.72–1.50)	1.19 (0.67–2.10)	0.51	10	0.77 (0.52–1.15)	0.86 (0.62–1.21)	0.51	0.15
Surface chemokine receptor expression on CD3$^+$ lymphocytes									
CCR1 (geometric mean expression)	9	5.04 (4.44–5.73)	6.21 (5.29–7.29)	0.01	10	4.78 (4.17–5.49)	4.83 (3.80–6.15)	0.88	0.85
CXCR3 (geometric mean expression)	9	8.46 (7.13–10.04)	11.02 (8.34–14.55)	0.01	10	8.52 (6.45–11.24)	9.12 (5.83–14.27)	0.72	0.97
CCR5 (geometric mean expression)	9	4.77 (4.03–5.65)	6.02 (4.76–7.61)	0.03	10	4.66 (3.91–5.55)	5.76 (4.34–7.64)	0.07	0.91
Supernatant levels after 36 h PHA									
IFNγ (ng/mL)	10	5.99 (2.98–12.07)	3.80 (1.84–7.87)	0.33	9	1.37 (0.45–4.16)	3.89 (2.09–7.24)	0.26	0.02
IL-2 (ng/mL)	10	13.10 (7.22–23.78)	11.79 (7.12–19.51)	0.14	9	11.96 (7.69–18.62)	12.98 (8.34–20.20)	0.33	0.46
TNFα (ng/mL)	10	1.45 (0.86–2.46)	1.45 (0.99–2.13)	0.65	8	1.26 (0.71–2.26)	1.75 (0.90–3.38)	0.03	0.72
IL-4 (pg/mL)	10	99.7 (55.4–179.7)	117.6 (75.6–182.9)	0.76	9	69.0 (36.5–130.6)	54.0 (23.0–126.9)	0.37	0.45
IL-5 (pg/mL)	10	86.1 (45.0–164.7)	86.2 (61.3–121.1)	0.86	9	58.1 (47.2–71.6)	89.4 (50.9–157.1)	0.13	0.84
IL-10 (ng/mL)	9	4.90 (3.43–7.01)	4.73 (3.00–7.45)	0.67	9	4.78 (3.28–6.97)	4.04 (2.88–5.67)	0.68	0.60
IFNγ/IL-4 ratio	10	60.1 (26.6–135.9)	32.3 (15.9–65.8)	0.14	9	19.9 (7.3–54.4)	72.0 (24.4–212.4)	0.008	0.06

Data are the mean ± SD or the geometric mean (95% CI of the mean). Adiol G, 5α-Androstane-3α,17β-diol glucuronide; DHEAS, dehydroepiandrosterone sulfate; F → M, female to male; IL-2, interleukin-2; IFNγ, interferon-γ; M → F, male to female; TNFα, tumor necrosis factor-α. Chemokine receptor expression are recorded as geometric means of histograms by FACS analysis. *P* values were assessed by Wilcoxon signed ranks tests for paired samples.

[a] *P* value by *t* test for paired samples.

[b] *P* value by *t* test for independent samples, for comparing baseline data of biological males to females.

[c] M → F transsexuals were treated with ethinyl estradiol, which cannot be detected in conventional 17β-estradiol assays.

from 20–70 yr (8, 9)], who had been treated with cross-sex hormones for 5 yr or more. The mean duration of estrogen administration was 12 yr (range, 5–32), and the mean duration since the sex reassignment surgery (*i.e.* orchidectomy) was 10 yr (range, 3–25). Current treatment consisted of oral ethinyl estradiol (Lynoral, 12.5–150 μg/day; n = 114), transdermal 17β-estradiol (25–100 mg twice per week; Estraderm TTS, Novartis, Basel, Switzerland; n = 37), oral conjugated estrogens (Premarin, Wyeth-Ayerst, Philadelphia, PA; or Dagynil, Dagra, Pharma, Diemen, The Netherlands; 0.625–2.5 mg/day; n = 26), oral 17β-estradiol (Progynova, Schering AG; 1–4 mg/day; n = 7), or parenteral 17β-estradiol (Progynon-depot, Schering AG, Berlin, Germany; 10/2 weeks; n = 2). Some combined their treatment with the antiandrogen cyproterone acetate (Androcur, Shering; 2–100 mg/day; n = 47) or spironolactone (Aldactone, Searle, Chicago, IL; 100–200 mg/day; n = 3). For every M→F transsexual, a control male was selected of similar age (±2 yr), with a median age of 42 yr (range, 20–72); they were M→F transsexuals before hormone administration (n = 131) and healthy nontranssexual males (n = 55). Male controls were eugonadal by clinical and laboratory criteria: testosterone, 20 ± 7 nmol/L (mean ± SD); 17β-estradiol, 86 ± 24 pmol/L; and LH, 3.1 ± 1.6 U/L. Serum PRL levels, available in 129 M→F transsexuals and 131 male controls, were used as an index of estrogen administration. Serum TPO-Ab titers were assayed using the Mileniatest (Diagnostic Products) and titers of 10 IU/mL or more were considered positive.

Statistical analysis

Variables with distributions that were skewed to the right (cytokine levels and expression, chemokine receptor expression, Ig isotype levels) were logarithmically transformed before analysis to normalize their distributions. Antilogarithms of the transformed means were used to obtain geometric means and 95% confidence intervals (CI) of the means. Mann-Whitney tests for independent samples were used to compare baseline differences. Wilcoxon's signed ranks test was used to analyze the effects of cross-sex hormones after 4 months for all measurements as well as for comparisons of PRL and TPO-Ab levels between M→F transsexuals and male controls. Spearman's correlation coefficients were used for intercorrelations. In the M→F and the F→M groups separately, an ANOVA for repeated measurements was used to analyze the effects of cross-sex hormones at three time points on Ig (sub)class levels as well as to compare at two time points the effects of estrogens plus antiandrogens with those of androgens. If values were below the lower limit of detection, the value of that lower limit was used for statistical calculations (for 17β-estradiol, 90 pmol/L; for testosterone, 1.0 nmol/L; for 5α-dihydrotestosterone, 0.1 nmol/L; for LH, 0.3 IU/L; for FSH, 0.5 IU/L; for IgE, 2 U/mL). A two-tailed *P* value of less than 0.05 was considered statistically significant. The software used was SPSS for Windows 8.0 (SPSS, Inc., Chicago, IL).

Results

Endocrine measurements

After estrogen plus antiandrogen administration to M→F transsexuals, serum levels of total testosterone, 5α-dihydrotestosterone, Adiol G, DHEAS, LH, and FSH were significantly suppressed, mostly to undetectable levels. The ethinyl estradiol that had been administered could not be detected by the assay used, but there were clear physical signs of estrogen effects in these subjects. Free cortisol excretion in 24-h urine samples and serum PRL levels showed a significant increase, whereas serum GH levels slightly increased (Table 1).

After parenteral testosterone administration to F→M transsexuals, the serum levels of total testosterone, 5α-dihydrotestosterone, and Adiol G significantly increased, whereas serum levels of 17β-estradiol, LH, and FSH levels were slightly, but significantly, suppressed. Serum levels of DHEAS, PRL, and GH as well as free cortisol excretion in 24-h urine samples did not change significantly (Table 1).

Leukocytes and lymphocyte subpopulations

After estrogen plus antiandrogen administration to males, the mean number of leukocytes increased, whereas no change occurred after testosterone administration to females. There was a tendency toward a lower number of $CD4^+$ T cells ($P = 0.052$) and a lower $CD4^+/CD8^+$ ratio ($P = 0.08$) in males compared to females (Table 1). Upon cross-sex hormone administration there were no changes in either the total number of lymphocytes or the relative number of $CD4^+$ and $CD8^+$ T cells in male or in female transsexuals (Table 1 and Fig. 1). After estrogen plus antiandrogen administration to males, both absolute (data not shown; $P = 0.03$) and relative numbers of NK cells decreased ($P = 0.01$), whereas a slight increase was found in the absolute (data not shown; $P = 0.24$) and relative number ($P = 0.11$; Table 1 and Fig. 1) of B cells. Upon testosterone administration to females, changes in NK and B cells, were less obvious and did not reach statistical significance (Table 1 and Fig. 1). However, the effects of

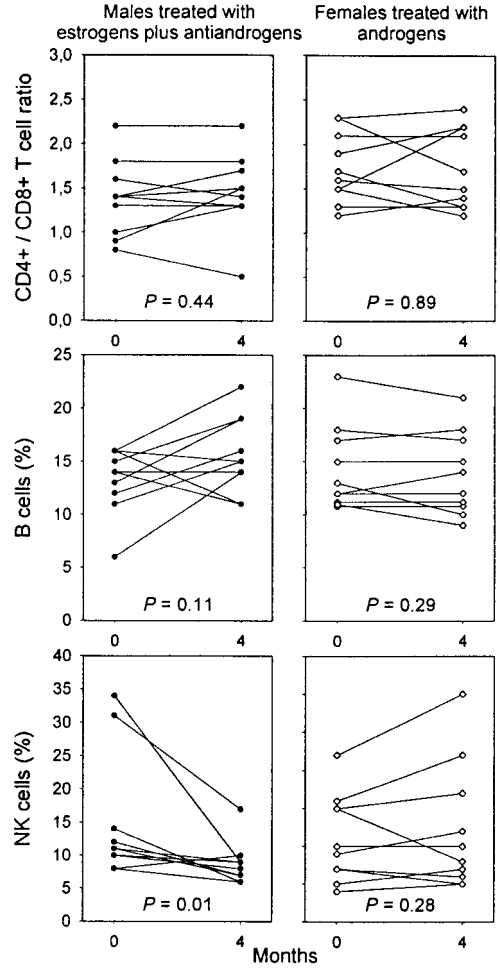

FIG. 1. Individual values on logarithmic scales of the $CD4^+/CD8^+$ T cell ratio and the number of B cells ($CD19^+$) and NK cells ($CD16^+$ or $CD56^+/CD3^-$), relative to the total number of lymphocytes. Lymphocytes were obtained from 10 M→F transsexuals receiving estrogens and antiandrogens and from 10 F→M transsexuals receiving androgens at baseline and after 4 months of hormone administration. *P* values were assessed by Wilcoxon signed ranks tests for paired samples.

estrogen plus antiandrogen were the opposite of those of testosterone ($P = 0.02$ for NK cells and $P = 0.07$ for B cells, for differences between M→F and F→M transsexuals by ANOVA for repeated measurements).

Production and intracellular expression of cytokines

Before hormone administration, males produced higher levels of IFNγ upon PHA stimulation than females ($P = 0.02$). The number of IFNγ-producing T cells also tended to be higher in males ($P = 0.07$; Table 1). Upon hormone administration, no significant differences were found for individual cytokines, either in supernatants upon PHA stimulation or by intracellular staining, except for the PHA-stimulated production of TNF-α, which increased significantly upon testosterone administration ($P = 0.03$; Table 1). However, an overall shift toward a T_H1 phenotype, as evaluated by the PHA-stimulated production ratio of IFNγ to IL-4, was found in females upon testosterone administration ($P = 0.008$), whereas this T_H1 phenotype tended to be down-regulated in males upon estrogen and antiandrogen administration ($P = 0.14$ and $P = 0.001$ for the difference between M→F and F→M transsexuals in an ANOVA for repeated measurements; Table 1 and Fig. 2).

Chemokine receptor expression

To investigate to what extent the predominant T_H1 profile found in males would be reflected by phenotypic analyses of

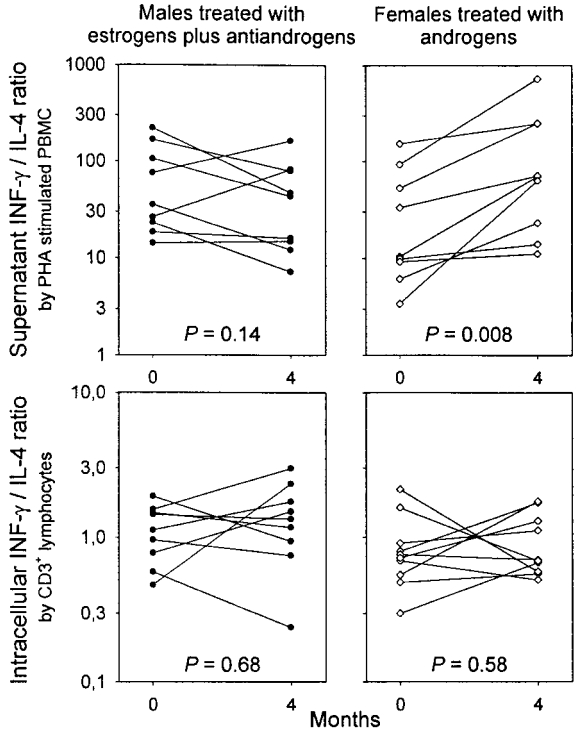

FIG. 2. Individual values on logarithmic scales of the IFNγ/IL-4 ratios. Lymphocytes were obtained from M→F transsexuals receiving estrogens and antiandrogens and from F→M transsexuals receiving androgens at baseline and after 4 months of hormone administration. Cytokine production was measured either in supernatants upon PHA stimulation or by intracellular staining upon phorbol myristate acetate/ionophore stimulation. *P* values were assessed by Wilcoxon signed ranks tests for paired samples.

T_H1-associated chemokine receptors on the lymphocyte surface, we studied baseline and hormone-induced changes in CCR1, CXCR3, and CCR5 expression. Before hormone administration, no sex differences were found in these chemokine receptor profiles. At baseline, inverse, but weak, correlations were found between the geometric mean surface expression of these chemokine receptors and the IFNγ/IL-4 ratio in both PHA-stimulated supernatants and cytokine-producing T cell numbers (Table 2).

After estrogen plus antiandrogen administration, but not after testosterone administration, the mean expression of CCR1, CXCR3, and CCR5 increased significantly in both $CD8^+$ and $CD8^-$ T cells (Table 1 and Fig. 3). The fraction of $CD3^+$ T cells expressing CCR1 increased from 2.9% to 5.4%, the fraction that expressed CXCR3 increased from 20.7% to 27.9%, and the fraction that expressed CCR5 increased from 11.9% to 17.9%. After androgen administration to women, no significant effects were found (Table 1 and Fig. 3).

The surface expressions of CCR1, CXCR3, and CCR5 were strongly and positively intercorrelated (Table 2). Analysis of their individual proportional changes after 4 months showed that, similar to baseline values, these changes were inversely correlated with the T_H1 phenotype, as evaluated by IFNγ/IL-4 production and intracellular expression ratio by T cells (Table 2 and Fig. 4). Concerning CCR5 expression by the helper subset of T cells, the proportional changes correlated inversely not only with the IFNγ/IL-4 ratio, but also with the numbers of TNFα-producing cells (data not shown).

Ig (sub)class levels

At baseline, significantly higher serum IgG and IgM levels were found in females compared to males (Table 1). Serum IgG4 and IgE levels were significantly correlated ($r = 0.33$; $P = 0.02$). After 4 months of estrogen plus antiandrogen administration to males, only the serum IgA level and the IgG4/IgG ratio decreased slightly (Table 1). After 4 months of androgen administration to females, serum IgA levels decreased also, but serum IgM levels increased (Table 1). The effects of estrogen plus antiandrogen and androgen treatment were not different after 4 or 12 months when comparing 13 M→F to 13 F→M transsexuals with complete data (for all, $P \geq 0.10$ in ANOVAs for repeated measurements), except for IgE levels ($P = 0.03$), which decreased in M→F after 12 months and did not change in F→M transsexuals ($P = 0.04$ and $P = 0.31$, respectively; 12 month data not shown). Sex differences in IgG and IgM were not essentially affected after cross-sex hormone administration (Fig. 5). Proportional individual changes after 4 months in serum levels of IgM, IgA, and IgG correlated strongly and positively (for all, $P < 0.001$).

TPO-Ab titers

Figure 6 shows the cross-sectional data on serum levels of PRL and TPO-Ab in 186 M→F transsexuals and 186 age-matched male controls. As expected, significantly higher PRL levels were found in M→F transsexuals compared to the male controls ($P < 0.001$, by Wilcoxon's signed ranks test). Fifteen M→F transsexuals as well as 15 male controls (8.0% for both) had a TPO-Ab titer of 10 U/mL or more. Also, when TPO-Ab levels in M→F transsexuals were compared to their

TABLE 2. (Inter)correlations of chemokine receptor expression on T cells and IFNγ/IL-4 ratios for baseline values and proportional individual changes after 4 months of hormone administration

	CXCR3	CCR5	Supernatant IFNγ/IL-4 production ratio	Intracellular IFNγ/IL-4 expression ratio
Baseline data				
Males and females pooled				
CCR1	0.21 (0.38)	0.55 (0.01)	−0.42 (0.08)	−0.44 (0.05)
CXCR3		0.75 (<0.001)	−0.38 (0.11)	−0.32 (0.16)
CCR5			−0.37 (0.12)	−0.37 (0.11)
Supernatant IFNγ/IL-4 production ratio				0.33 (0.17)
	CXCR3[a]	CCR5[a]	Supernatant IFNγ/IL-4 production ratio[a]	Intracellular IFNγ/IL-4 expression ratio[a]
Prospective data				
M → F transsexuals				
CCR1 (% of pretreatment value)	0.37 (0.33)	0.70 (0.04)	−0.17 (0.67)	0.13 (0.73)
CXCR3 (% of pretreatment value)		0.88 (0.002)	−0.77 (0.02)	−0.75 (0.02)
CCR5 (% of pretreatment value)			−0.57 (0.11)	−0.40 (0.29)
Supernatant IFNγ/IL-4 production ratio (% of pretreatment value)				0.80 (0.01)
F → M transsexuals				
CCR1 (% of pretreatment value)	0.08 (0.83)	0.75 (0.01)	−0.23 (0.55)	−0.35 (0.33)
CXCR3 (% of pretreatment value)		0.33 (0.35)	−0.57 (0.11)	−0.08 (0.83)
CCR5 (% of pretreatment value)			−0.05 (0.90)	−0.41 (0.24)
Supernatant IFNγ/IL-4 production ratio (% of pretreatment value)				−0.13 (0.73)

Values are standardized Spearman's correlation coefficients (ρ), with P value in *parentheses.* F→M, Female to male; IL-4, interleukin-4; IFNγ, interferon-γ; M→F, male to female.

[a] Values are expressed as a percentage of the pretreatment value.

matched control levels, no significant difference was found ($P = 0.23$, by Wilcoxon's signed ranks test; Fig. 6). The duration of estrogen administration was not associated with TPO-Ab levels in M→F transsexuals (data not shown). Compared to the age-adjusted prevalences of TPO-Ab in a previous study (9) a significant difference was found between M→F transsexuals and females (8.0% *vs.* 14.1%; $P < 0.001$, by χ^2 test), but no significant difference was found between M→F transsexuals and males (8.0% *vs.* 6.4%; $P = 0.13$, by χ^2 test).

Discussion

This study of transsexuals undergoing cross-sex hormone treatment, with radical changes in their sex steroid milieus, focused on the effects of sex steroids on parameters of lymphocyte function and serum levels of Ig (sub)classes and TPO-Ab. One of the major phenotypical effects of estrogen administration was the reduction of NK cells numbers. NK cell cytotoxicity is also inhibited by the use of oral contraceptives (45) and during pregnancy, and is inversely associated with serum 17β-estradiol levels (46). *In vitro,* NK cell cytotoxicity is inhibited by diethylstilbestrol (47, 48) and enhanced by the estrogen receptor antagonist tamoxifen (49). NK cells are essential during host defenses against tumor and viral targets and are believed to prevent the development of autoantibodies by production of the immune system suppressor, transforming-growth factor-β. The differential effects of estrogens plus antiandrogens *vs.* testosterone on the number of NK cells and B cells might explain part of the sex difference in the prevalence of various systemic autoimmune diseases (2). The latter pathologies and especially systemic lupus erythematosus seem to be associated with impaired NK cell activity and B cell hyperactivity (50). Data from animal studies suggested that estrogens enhance $CD4^+$ T cell function, and androgens enhance $CD8^+$ T cell function (51); testosterone administration increased the number of $CD8^+$ T cell in men with Klinefelter's syndrome (52). Nevertheless, we did not find any change in the number of $CD4^+$ and $CD8^+$ T cells.

TPO-Ab were chosen as an example of autoantibodies with a relative high prevalence in females, *i.e.* 13% compared to 6.1% in males (9). Although estrogen treatment and castration of male animals result in increased autoantibody levels in murine models (21–25), we found a similar frequency (8%) and similar titers of TPO-Ab in orchidectomized males on long term estrogen treatment and in age-matched, male controls. Hyperprolactinemia, induced by long term estrogen therapy and found to be related to TPO-Ab in a previous study (53), was not associated with elevated TPO-Ab levels. Reports on the *in vivo* effects of endogenous and exogenous estrogens on IgA, IgG, and IgM levels conflict (54–56). In the present study we confirm the finding of higher IgG and IgM levels at baseline in women compared to men (3–6). Upon cross-sex hormone administration, however, only minor changes could be observed after 4 and 12 months, and the sex differences before treatment were not reversed. Moreover, the effects on IgA, IgG, and IgM levels of estrogen plus antiandrogen administration in men were largely similar compared to those of testosterone administration in women. Administration of sex steroids may cause body water retention (57, 58), and the increased plasma volume may have diluted the concentrations of circulating Igs in both treatment

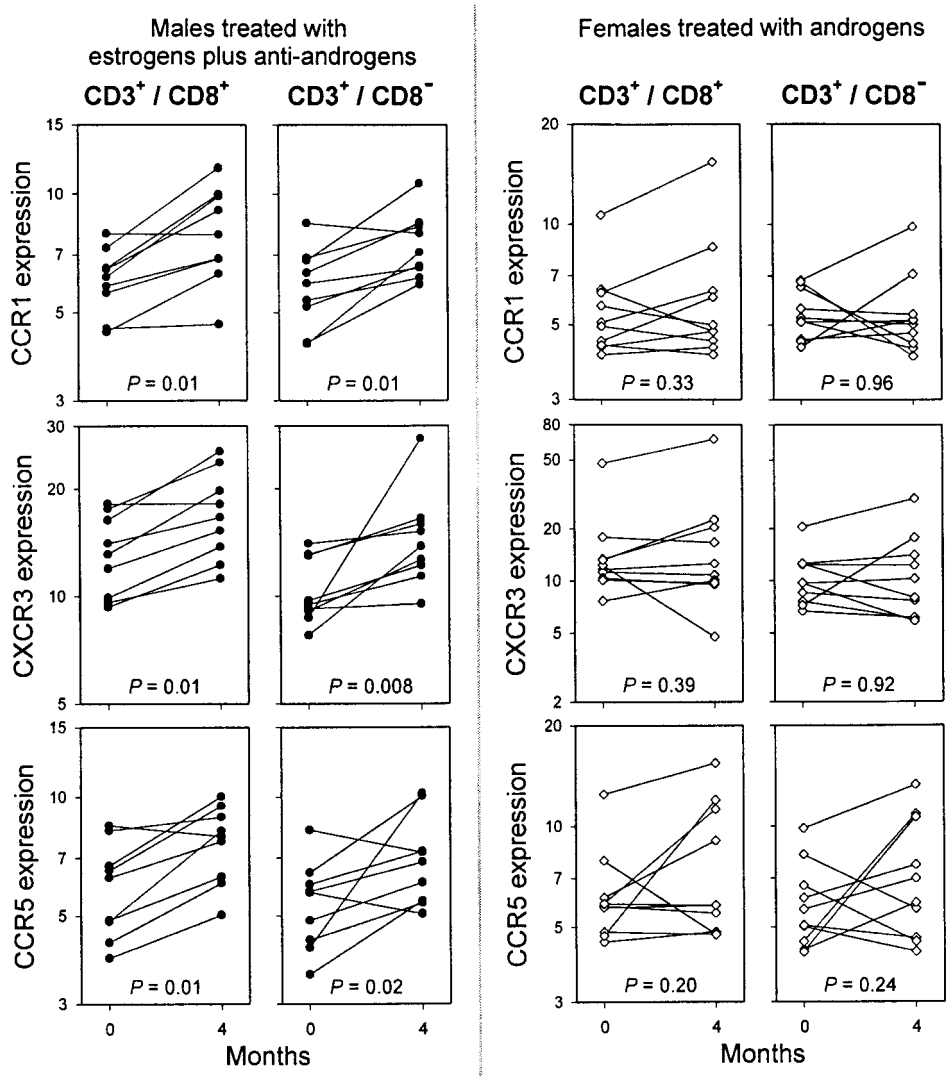

FIG. 3. Individual values on logarithmic scales of CCR1, CXCR3, and CCR5 expression on the $CD8^+$ and $CD8^-$ T cell surface. Lymphocytes were obtained from 9 M→F transsexuals receiving estrogens and antiandrogens and from 10 F→M transsexuals receiving androgens at baseline and after 4 months of hormone administration. Chemokine receptor expression was recorded as geometric means of histograms by FACS analysis. *P* values were obtained by Wilcoxon signed ranks tests for paired samples.

groups. This concept is supported by the strong positive correlations between changes in IgA, IgG, and IgM levels.

Our data showed that testosterone administration increased the IFNγ/IL-4 production ratio of peripheral blood T cells in women, whereas estrogens plus antiandrogens slightly decreased this ratio in men. Differential effects of sex steroids on cytokine production profiles may be of clinical importance for the outcome of immune-mediated inflammatory disease. For example, the higher risk of developing systemic lupus erythematosus (59) and the increased number of flares (60) in response to pregnancy or estrogen use may be due to potentiation of the T_H2 pathway by estrogens. Estrogen-induced inhibition of T_H1 responsiveness against human papillomavirus infections (61) in women using oral contraception could partially explain the epidemiological association among oral contraception, urogenital infection, and cervical cancer (16). The risk of cardiovascular disease, preceded by infiltration of macrophages and T cells in the blood vessel wall (62), may be reduced in women who receive estrogen therapy (63). It could be hypothesized that a reduced IFNγ production may contribute to this reduced cardiovascular risk, as illustrated by the reduction of atherosclerotic lesions in IFNγ receptor knockout mice (64).

As phenotypic markers for T_H1-like T cells, we assessed the expression of the chemokine receptors CCR1, CXCR3, and CCR5 (32–35). Unexpectedly, upon estrogen plus antiandrogen administration, but not upon testosterone administration, these chemokine receptors were up-regulated on T cells, which consequently may affect their ability to migrate. Although proinflammatory cytokines can stimulate chemokine production and chemokine receptor expression *in vitro* (30, 65, 66), our *in vivo* data suggested that CCR1, CXCR3, and CCR5 expression is inversely associated with the T_H1/T_H2 balance. In fact, negative correlations were found in PBMC suspensions tested between CCR1, CXCR3, and CCR5 expression and the ratio of IFNγ to IL-4. However, this phenomenon could not be evaluated at the single cell level, because the expressions of chemokine receptors and cytokines were not determined in a double staining. Apparently, CCR1, CXCR3, and CCR5 expression does not provide a simple phenotypic marker for PBMC propensity toward T_H1 cytokine production. The increased CCR5 expression on

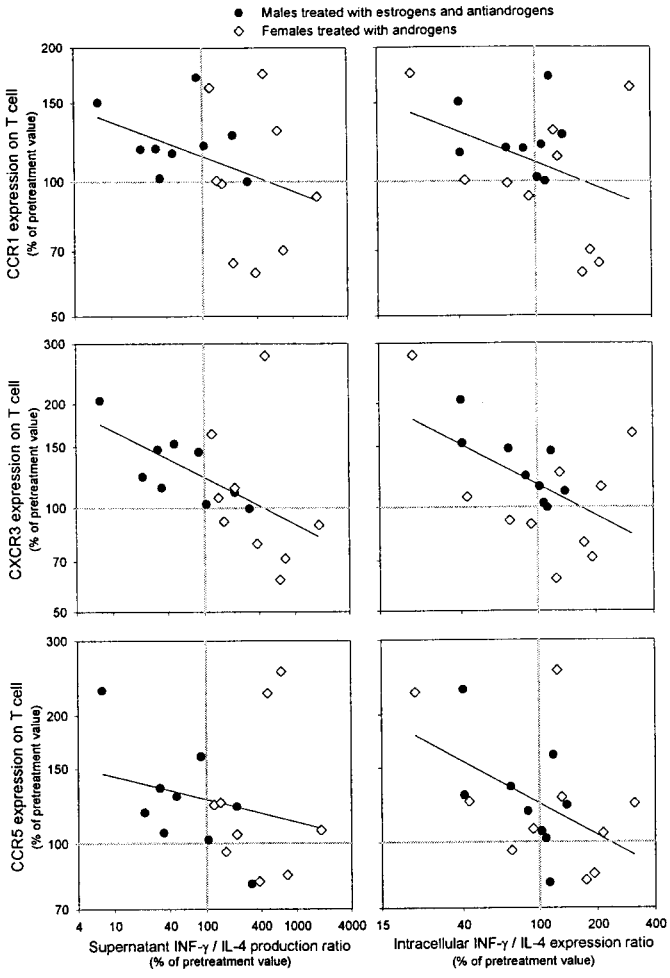

FIG. 4. Associations between the individual proportional changes, at 4 months *vs.* baseline, of CCR1, CXCR3, and CCR5 expression and those of the IFNγ/IL-4 ratios. Chemokine receptor expression was recorded as geometric means of histograms by FACS analysis. Univariate regression lines in pooled subjects are shown on logarithmic scales. Correlation coefficients and *P* values, for M→F and F→M transsexuals separately, are presented in Table 2.

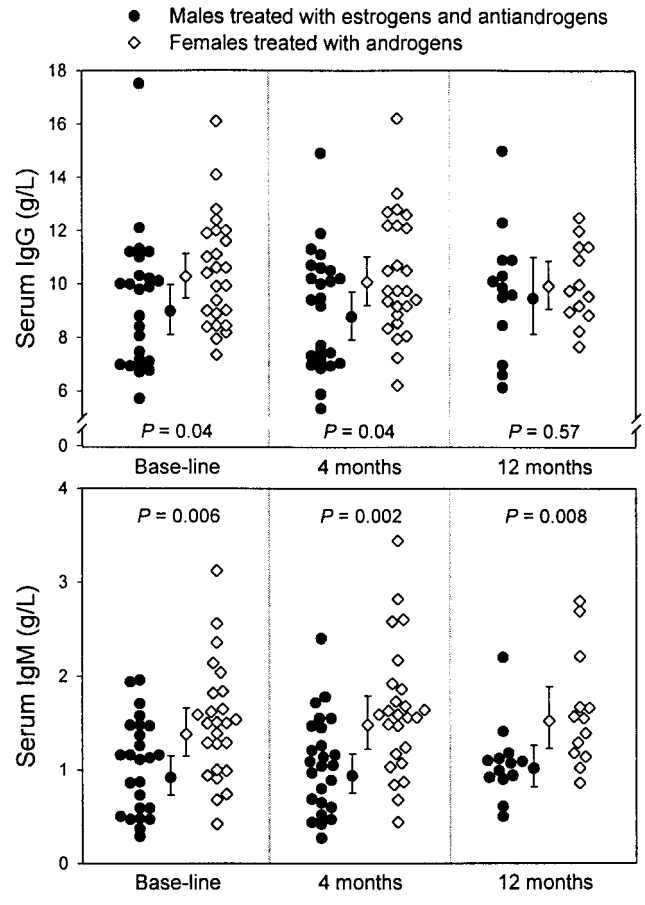

FIG. 5. Individual levels of IgG and IgM (with error bars representing geometric mean and 95% confidence interval of the mean). Serum were obtained from 25 M→F and 25 F→M transsexuals at baseline and after 4 months of cross-sex hormone administration, and of 13 M→F and 13 F→M transsexuals at 12 months. *P* values were assessed by Student's *t* tests using logarithmically transformed data, comparing biological males and females.

helper T cells (11.6% to 18.1%) upon estrogen plus antiandrogen administration may have some implications for AIDS pathogenesis (66), because CCR5 serves as coreceptor for the entry into macrophages and activated T cells of the macrophage-tropic strain of human immunodeficiency virus-1 (30, 67).

The observed changes in immune parameters may be caused directly by the administered hormones themselves or may be secondary to complex paracrine responses from adjacent cells and mechanisms affecting other hormones, such as adrenal hormones. We noted in this study that free cortisol, known for its antiinflammatory T_H1-suppressive properties (68), was increased in 24-h urine samples after estrogen plus antiandrogen administration and was slightly decreased after testosterone administration. Especially estrogens may induce a hyperresponsiveness of the hypothalamic-pituitary-adrenal axis (69, 70). Expected effects on the secretion of norepinephrine and epinephrine as well as observed effects on cortisol (70), as part of the stress system, could explain part of the observed effects on immune parameters. Additionally, in present and previous (71, 72) studies of cross-sex hormone administration, we observed changes in dehydroepiandrosterone, PRL, GH, insulin-like growth factor I, LH, and FSH, all of which have potentially immunostimulatory properties (41, 73–75). Especially, the effects of testosterone may be indirect through changes in other hormones or via dendritic cells (76), because T cells do not contain testosterone-binding sites (14, 15).

The interpretation of our results is limited by the inclusion of a relatively small number of subjects in the prospective part of the study and the lack of a placebo group due to the nature of the study population and the treatment indication. Furthermore, the effects of sex steroids in humans depend on the route of administration and the dosage. Our study assessed the effects of androgens in women and of estrogens plus antiandrogens in men. We cannot be sure whether sex-appropriate sex steroids would have had similar effects. To date, effects of sex steroids on immune responsiveness have only been studied *in vitro* (17, 18, 39, 41, 42). To the best of our knowledge, this is the first experimental study in humans reporting on overall *in vivo* effects.

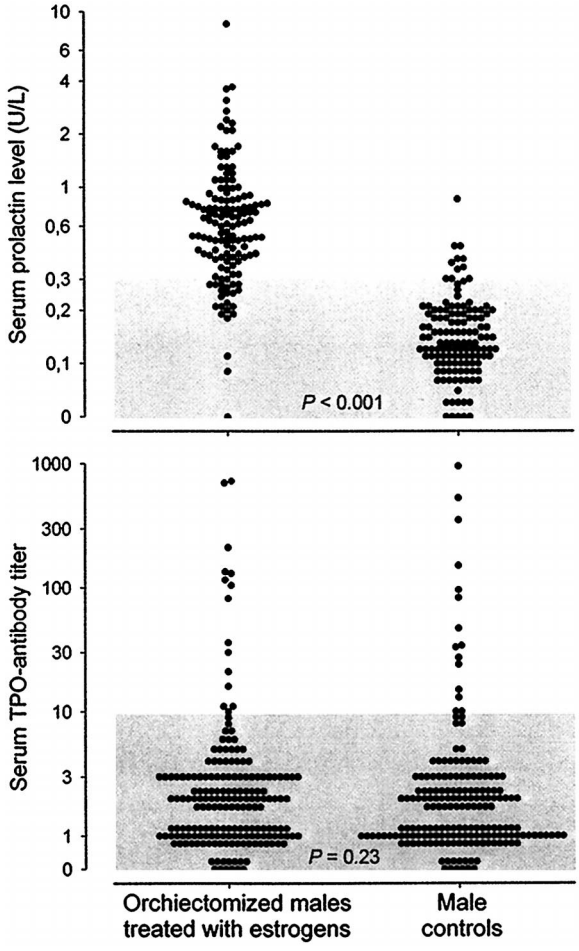

FIG. 6. Serum PRL levels and TPO-Ab on a logarithmic scale in 186 M→F transsexuals, after orchidectomy (*i.e.* castration operation) and treatment with estrogens for 5 yr or more compared to 186 age-matched male controls. The reference range for PRL in men is less than 0.3 U/L. Fifteen M→F transsexuals had a TPO-Ab titer of 10 U/mL or more, which was considered positive compared to that in 15 male controls. *P* values were assessed by Wilcoxon signed ranks tests for paired samples, comparing each M→F transsexual with her matched male control.

In conclusion, estrogens plus antiandrogens in men decreased the number of NK cells and up-regulated the expression of T_H1-associated chemokine receptors. In addition, our data provide evidence for a role of sex steroids as one of the many signals involved in regulation of the T_H1/T_H2 balance in men and women and suggest that testosterone drives peripheral blood T cells down a T_H1 pathway, whereas estrogens may skew differentiation toward T_H2. These changes may be direct or indirect through the effects on other hormones, especially those of the hypothalamic-pituitary-adrenal axis.

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