

CLINICAL STUDY

Consistent production of a higher T_H1:T_H2 cytokine ratio by stimulated T cells in men compared with women

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Abstract

Objective: To evaluate the T helper 1 (T_H1)/T helper 2 (T_H2) lymphocyte cytokine profiles in women and men and to study the *in vitro* effects of sex hormones on lymphocyte secretion of cytokines.

Methods: Analysis of serum concentration and lymphocyte synthesis of T_H1 (gamma interferon (INF- γ) and interleukin 2 (IL-2)) and T_H2 (interleukin 4 (IL-4) and interleukin 10 (IL-10)) cytokines was performed in 20 women and 15 men. Analysis of modifications in cytokine secretion induced by supplementation of lymphocyte culture with increasing concentrations of sex hormones was carried out.

Results: Higher levels of INF- γ and IL-2 and lower levels of IL-4 and IL-10 were detected in the phytohemagglutinin-stimulated lymphocyte culture supernatants of men compared with women; the INF- γ :IL-4 ratio was significantly higher in men. In women, similar concentrations of all the cytokines were detected in culture supernatants obtained during the follicular and the luteal phases. The addition of sex hormones did not modify the concentration of cytokines in supernatants of phytohemagglutinin-stimulated T-cell cultures.

Conclusions: Women present a predominant T_H2 cytokine profile, which could be involved in immune responses characterized principally by the secretion of antibodies. This could be a factor implicated in the higher concentration of immunoglobulins or the increased prevalence of autoimmune diseases detected in females.

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Introduction

Evidence from mouse (1) and human (2) studies indicates that T helper cells can be divided into at least two functional subsets on the basis of their cytokine profiles. T helper 1 (T_H1) lymphocytes secrete predominantly interleukin 2 (IL-2) and gamma interferon (INF- γ) and thus play a role in the activation and recruitment of other T cells and macrophages; T helper 2 (T_H2) lymphocytes secrete interleukin 4 (IL-4), a cytokine which influences antibody production by B lymphocytes, and interleukin 10 (IL-10). T_H1 and T_H2 clones are reciprocally regulated by their secreted cytokines: INF- γ inhibits the proliferation of T_H2 clones whereas IL-10 inhibits that of T_H1 clones (3).

In recent years, the relationship between the immune and endocrine systems has been extensively analyzed (4). The presence of estrogen receptors on immune cells

has been demonstrated (5, 6), along with the modulation of hormonal processes by cytokines (7). Indirect evidence suggests that the cytokine profiles in women and men differ: (i) it has been demonstrated that estrogens can stimulate the secretion of immunoglobulins (Igs); moreover, serum Ig concentrations are higher in women than in men (8–10); (ii) women are more resistant to the induction of tolerance (10); and (iii) the prevalence of autoimmune diseases is higher in women (11). The differences described do not present themselves before puberty. These findings suggest a different regulation of the adult immune system as a function of gender, there being a preponderance of the T_H2 profile in women.

The aim of this study has been to analyze the serum concentration and lymphocyte secretion of T_H1 and T_H2 cytokines in women and men and the *in vitro* effects of sex hormones on this secretion.

Subjects and methods

Subjects

Twenty women (mean age 24.9 ± 4.9 years) and 15 men (mean age 28.1 ± 6.7 years) were recruited for the study. Subjects had to comply with the following conditions: (i) the absence of clinical evidence of disease and (ii) the absence of previous or current treatment.

A clinical history and a basic complementary evaluation (hemogram; determination of serum concentrations of creatinine, glucose and liver enzymes) were performed for every individual. Specifically, women were questioned about alterations in fertility or the menstrual cycle (12). The menstrual formula was 3–5/28–30. Men were also questioned about alterations of sexual characteristics or functioning (13). No abnormalities were detected in any of the subjects.

Informed consent was obtained from every subject. The study was approved by the Institutional Ethics Committee.

Study protocol

Venous blood was obtained from women on two days of their menstrual cycle: the 5th day (representative of the follicular phase) and the 25th day (representative of the luteal phase). Only one blood sample was obtained from each of the men.

Samples were obtained in every case before 0800 h to avoid circadian variations. Aliquots were used for (i) determination of serum concentrations of estradiol, progesterone and testosterone, (ii) analysis of serum concentrations of IgG, IgA and IgM, (iii) analysis of serum concentrations of cytokines, and (iv) selection of lymphocyte-enriched populations.

Sex hormones

Serum concentrations of estradiol, progesterone and testosterone were measured using enzyme immune assay kits (Enzymum-test, Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's instructions. Normal serum concentrations of these hormones in our laboratory, obtained after testing a total of 500 samples from healthy blood donors, are as follows: estradiol: follicular phase, 37–540 pmol/l; luteal phase, 100–900 pmol/l; men, <145 pmol/l; progesterone: follicular phase, <5 nmol/l; luteal phase, 5.5–65 nmol/l; men, <5 nmol/l; testosterone: women, <3.5 nmol/l; men, 7–30 nmol/l.

Serum Igs

Serum concentrations of Igs were determined by nephelometry (Behringwerke AG, Marburg, Germany). The normal ranges of Ig concentrations, obtained after testing a total of 500 samples from healthy blood donors, were as follows: IgG, 800–1500 mg/l; IgA,

90–315 mg/l; and IgM, 45–150 mg/l. Our reference values match those provided by the manufacturer (Behringwerke AG, Marburg, Germany).

Selection of lymphocyte-enriched populations and cell-culture conditions

Peripheral blood mononuclear cells were obtained using the Ficoll gradient method. Lymphocyte-enriched populations were obtained after double rosetting, as previously described (14). The purity of the population, as determined by CD3+ cell count, was in every case higher than 95%. After being counted, cells were resuspended in RPMI 1640 (Flow Laboratories, Irvine, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA), 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM) and 1% penicillin–gentamicin, and checked for viability by trypan blue exclusion.

Cytokine-enriched supernatants were obtained by culturing T cells at 37 °C in a humidified atmosphere containing 5% CO₂, at a density of 5×10^6 cells/ml in complete medium. Cultures were incubated in the presence or absence of phytohemagglutinin (10 µg/ml), and supernatants were harvested at 1, 2 and 3 days incubation, sterilized by filtration through a 0.22-µm filter (Millipore Company, Bedford, CA, USA) and stored at –70 °C until used. Phytohemagglutinin was tested in dose/response titrations before use. Time-response curves showed that maximum synthesis of cytokines was detected at 24 h of culture; thus, henceforth only data from this time of culture will be presented.

In a set of experiments, T cells from five men and five women were cultured, in the conditions mentioned above, in the presence of various (including physiological) concentrations of sex hormones: estradiol, 30, 300 and 3000 pmol/l; progesterone, 5, 50 and 500 nmol/l; and testosterone, 5, 50 and 500 nmol/l. Hormones were provided by Sigma (St Louis, MO, USA).

Determination of cytokine levels

Serum and culture supernatant samples for cytokines were frozen at –70 °C and thawed only once. All of the specimens were coded, allowing experimental procedures to be performed blind. Concentrations of cytokines were measured with enzyme-linked immunological binding assays (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Each sample was assayed in duplicate. Detection limits were as follows: INF-γ, 3.0 pg/ml; IL-2, 7.0 pg/ml; IL-4, 2.0 pg/ml; IL-6, 30 pg/ml; and IL-10, 5.0 pg/ml. All samples obtained from each subject were assayed in the same run. The coefficient of inter-assay variation oscillated between 4.3 and 5.1% and the coefficient of intra-assay variation was between 2.9 and 4.8%. The INF-γ:IL-4 ratio was estimated as an index of the T_H1/T_H2 profile.

To exclude the existence of up-regulation of the immune system or interference from silent infections, we also measured serum levels of IL-6, a cytokine which is neither strongly T_H1- nor T_H2-related (3).

Statistical analysis

Data are presented as means $\pm$ standard deviations, medians (ranges) or, when indicated, as absolute numbers and percentages. The data from two independent groups were compared using the Mann–Whitney U test. The significance of parameters within each group was tested by the Wilcoxon matched-pairs signed rank test. For qualitative variables, χ^2 with Yates' correction or Fisher's exact test was used. Standard regression analysis and Pearson rank correlation coefficients were used to identify the relationship between serum or supernatant concentrations of cytokines and serum concentrations of sex hormones or Igs. All analyses were done using SPSS (Statistical Package for Social Studies) software, version 5.0 (SPSS Inc., Chicago, IL, USA).

Results

The results of hormonal determinations are provided in Table 1. Evaluation of both vaginal epithelium and serum concentrations of progesterone during the luteal phase (in every woman higher than 16 nmol/l) established that the ovulatory cycles were normal.

The serum IgG concentration was similar in the follicular and the luteal phase, but was significantly higher in women than in men. Serum concentrations of Igs M and A were similar in women and men (Table 1). No significant correlation was obtained between serum concentrations of sex hormones and Igs (data not shown).

Serum concentrations of INF- γ , IL-2, IL-4 and IL-10 were below the level of detection of the assay. Serum concentrations of IL-6 were near the lower limit of the

assay, being similar in men (mean $\pm$ S.D.: 83.6 $\pm$ 38.3, median: 53, range: <30, 80 pg/ml) and in women (follicular phase, mean $\pm$ S.D.: 70.6 $\pm$ 99.5 median: 60, range: <30, 99 pg/ml; luteal phase, mean $\pm$ S.D.: 43.9 $\pm$ 48.6, median: 39.3, range: <30, 58 pg/ml) ($P > 0.05$).

Unstimulated synthesis of INF- γ , IL-2, IL-4 and IL-10 by peripheral blood lymphocytes was undetectable. After stimulation with phytohemagglutinin, similar concentrations of all the cytokines were detected in culture supernatants obtained during the follicular and the luteal phases. Although not significant, higher levels of INF- γ and IL-2 and lower levels of IL-4 and IL-10 were detected in the culture supernatants of men compared with women; the INF- γ :IL-4 ratio was significantly higher in men (Table 2).

With the objective of analyzing the possible modulation of the secretion of cytokines by sex hormones, lymphocytes obtained from five men and five women were cultured in the presence of increasing concentrations of estradiol, progesterone, estradiol plus progesterone, and testosterone. Again, spontaneous secretion of cytokines was undetectable, irrespective of the presence or absence of any hormones. The addition of sex hormones did not modify the concentrations of cytokines in supernatants of phytohemagglutinin-stimulated T cell cultures (Fig. 1).

Discussion

Our study has demonstrated the existence of differences in the T_H1/T_H2 profile between men and women. T_H1 and T_H2 clones are reciprocally regulated by their secreted cytokines: INF- γ inhibits the proliferation of T_H2 clones whereas IL-10 inhibits that of T_H1 clones (3). We have used, in our *in vitro* studies, a population of CD3 cells which include CD4 helper cells, CD8 cytotoxic cells and natural killer (NK) cells, all of which contribute to cytokine synthesis (15). However, a certain lymphokine pattern (INF- γ :IL-4 ratio) is generally used as being representative of a T_H1- or T_H2 profile (3) and

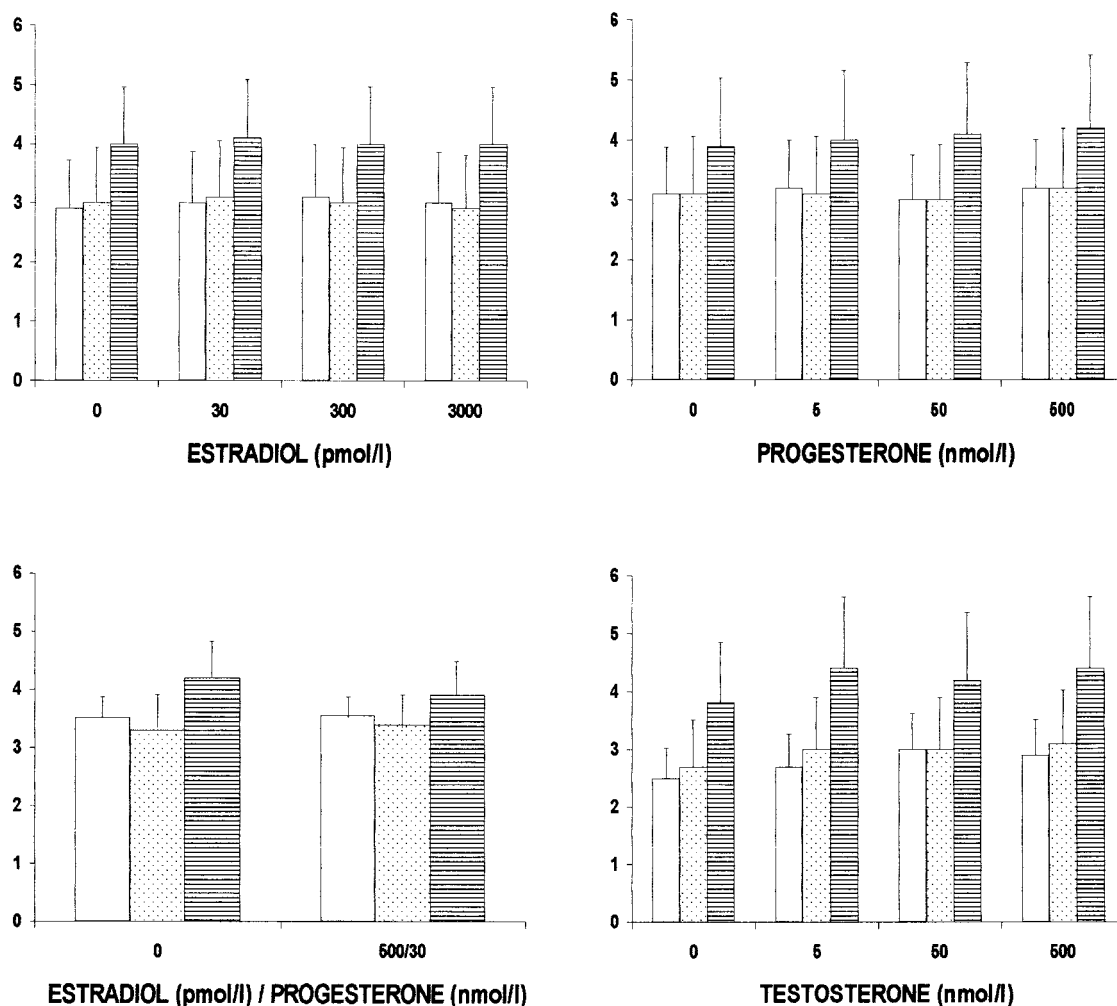
Table 1 Serum concentration of sex hormones and Igs in the study population.

Parameter	Women (n = 20)						Men (n = 15)		
	Follicular phase			Luteal phase					
	Mean $\pm$ S.D.	Median	Range	Mean $\pm$ S.D.	Median	Range	Mean $\pm$ S.D.	Median	Range
Sex hormone									
Estradiol (pmol/l)	152.7 $\pm$ 53.6	145	89–232	374.4 $\pm$ 109.8	348	345–786	107.6 $\pm$ 50.0	85	50–130
Progesterone (nmol/l)	0.6 $\pm$ 2.5	0.6	0.4–3.5	29.1 $\pm$ 10.6	26	15–34	0.3 $\pm$ 2.9	0.2	0.1–3.1
Testosterone (nmol/l)	3.0 $\pm$ 1.4	2.8	2.3–3.5	3.1 $\pm$ 1.0	2.8	1.9–3.4	26.2 $\pm$ 7.6	23	12–30
Immunoglobulin									
IgG (mg/dl)	1361 $\pm$ 114	1290	1120–1545	1316 $\pm$ 77	1270	1174–1490	1140 $\pm$ 68*	1136	895–1285
IgM (mg/dl)	119 $\pm$ 20	112	75–128	115 $\pm$ 31	113	70–132	127 $\pm$ 42	122	75–140
IgA (mg/dl)	197 $\pm$ 21	179	142–230	189 $\pm$ 19	168	136–220	217 $\pm$ 24	204	155–238

* $P < 0.05$ vs women, in both the follicular and luteal phases.

Table 2 Concentrations of T_H1 and T_H2 cytokines in supernatants of phytohemagglutinin-stimulated lymphocyte cultures obtained from peripheral blood from men and from women in both the follicular and luteal phases.

Parameter	Women (n=20)						Men (n=15)		
	Follicular phase			Luteal phase					
	Mean $\pm$ s.d.	Median	Range	Mean $\pm$ s.d.	Median	Range	Mean $\pm$ s.d.	Median	Range
INF- γ (pg/ml)	727.2 $\pm$ 163.0	612	325–1012	777.5 $\pm$ 125.7	543	341–785	889.3 $\pm$ 367.7	769	435–1320
IL-2 (pg/ml)	505.1 $\pm$ 138.2	390	312–754	521.0 $\pm$ 152.1	428	294–733	632.1 $\pm$ 176.6	545	390–1212
IL-4 (pg/ml)	258.3 $\pm$ 14.4	240	205–271	268.9 $\pm$ 28.4	251	196–271	193.4 $\pm$ 36.6	170	145–234
IL-10 (pg/ml)	235.3 $\pm$ 29.6	214	194–262	234.1 $\pm$ 19.7	206	194–270	189.7 $\pm$ 16.0	164	150–206
INF- γ /IL-4	2.8 $\pm$ 1.4*	2.6	1.7–3.7	2.9 $\pm$ 1.1†	2.3	1.9–3.5	4.6 $\pm$ 1.1*†	4.5	3.4–5.3

INF- γ , gamma interferon; IL-2, interleukin 2; IL-4, interleukin 4; IL-10, interleukin 10; INF- γ /IL-4, gamma interferon/Interleukin 4 ratio.* $P < 0.05$ vs women, follicular phase; † $P < 0.05$ vs women, luteal phase.**Figure 1** INF- γ :IL-4 ratio in supernatants of phytohemagglutinin-stimulated peripheral blood T-cell cultures from five women (obtained on the 5th (solid bars) and 25th (dotted bars) days of the menstrual cycle) and from five men (striped bars), in the presence of estradiol, progesterone, estradiol plus progesterone, and testosterone, at the concentrations indicated on the x-axis. Results are expressed as means $\pm$ standard deviations. Similar values for the INF- γ :IL-4 ratio were detected in the presence or absence of supplementary hormones, in both men and women ($P > 0.05$).

has been applied in this article. Although differences were not significant, we detected higher levels of INF- γ and IL-2, indicative of a T_H1 profile, in phytohemagglutinin-stimulated lymphocyte cultures from men compared with those from women. Moreover, the INF- γ :IL-4 ratio was significantly higher in men.

Sex hormones have been implicated in the modulation of several other immune functions. Specifically, it has been demonstrated that women in the luteal phase present an increased ability to synthesize monocyte-derived cytokines, such as interleukin 1 (IL-1) (16, 17). Synthesis of IL-1 has been correlated with serum concentrations of progesterone and it has been associated with the increase in body temperature in the luteal phase of the menstrual cycle. Moreover, physiological concentrations of progesterone, but not of estradiol, increased the monocyte synthesis of IL-1 *in vitro* (6). T-lymphocyte function can be also modified by estrogens or androgens. T-lymphocyte proliferative responses increase in the castrated animal; this increase can be reversed by the supplementation of T-cell cultures with estradiol or progesterone (9).

To evaluate the possible immunomodulatory action of sex hormones on lymphocyte secretion of cytokines, we supplemented lymphocyte cultures with various (including physiological) concentrations of estradiol, progesterone, estradiol plus progesterone, or testosterone (12, 13). However, the presence of supplementary hormones did not modify the concentration of secreted cytokines. It is possible that a genetic factor, and not an environmental factor such as the presence of sex hormones, could be operative. In fact, the pattern of secretion of cytokines in follicular and luteal phases, in which the serum concentrations of estradiol and progesterone are clearly different, was similar. Prolactin, the concentration of which is different in the two genders, has been demonstrated as being capable of modulating immune functions (18); this hormone may be a contributory factor in the absence of modulation of cytokine secretion by sex hormones.

It has been proposed that the T_H1/T_H2 balance is critical for driving specific types of immune response. A predominantly T_H1 profile, through cooperation with macrophages and cytotoxic T lymphocytes or NK cells, could be involved in the production of predominantly cytotoxic responses. In contrast, a predominant T_H2 profile is involved in an immune response characterized principally by the secretion of antibodies (3). In this respect, it has been demonstrated that estrogens stimulate Ig synthesis (8–10). Moreover, the higher concentrations of IgG detected in women compared with men (although both genders had values considered to be within the normal range) in our study and in previous studies (8–10) could be explained by the finding of a predominantly T_H2 cytokine profile in women.

Likewise, the relatively elevated T_H2 response could be implicated in a relatively poor cytotoxic response.

An increased T_H2 cytokine profile has been detected in systemic erythematous lupus (19, 20), primary biliary cirrhosis (21) and Graves' disease (22), all of which are considered to be autoimmune diseases. It is believed that autoimmune diseases, which as a group are more prevalent in women, are characterized by the persistence of the responsible (although unknown) antigen and polyclonal secretion of autoantibodies (11, 23). In addition to genetic factors, alterations in antigen presentation and exposure factors, a predominantly T_H2 cytokine profile could favor the development of such disease (24).

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