The immune response during the luteal phase of the ovarian cycle: a Th2-type response?

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Objective: To test the hypothesis that during the luteal phase of the ovarian cycle, as compared with the follicular phase, the peripheral immune response is shifted toward a type-2 response.

Design: Prospective study.

Setting: Academic research setting.

Patient(s): Women with regular menstrual cycles.

Intervention(s): Blood samples were collected between days 6 and 9 of the menstrual cycle and 6–9 days after the LH surge.

Main Outcome Measure(s): Intracellular cytokine production of interferon (IFN)-γ, interleukin (IL)-2, IL-4, and IL-10 after in vitro stimulation of lymphocytes as well as total white blood cell (WBC) count, differential WBC count, and plasma 17β-E_2 and P concentrations.

Result(s): Mean plasma 17β-E_2 and P concentrations, WBC count, and mean granulocyte, monocyte, and lymphocyte counts were significantly increased in the luteal phase as compared with the follicular phase of the ovarian cycle. Production of type-1 cytokines (IFN-γ and IL-2) and production of the type-2 cytokine IL-10 did not vary between the phases of the ovarian cycle. Production of the type-2 cytokine IL-4, however, was significantly increased in the luteal phase as compared with the follicular phase of the ovarian cycle.

Conclusion(s): During the luteal phase of the ovarian cycle, the immune response is shifted toward a Th2-type response, as reflected by increased IL-4 production in this phase of the cycle. These results may suggest that increased levels of P and 17β-E_2 in the luteal phase of the ovarian cycle play a role in the deviation of the immune response toward a type-2 response. (Fertil Steril® 2000;74:1008–13. ©2000 by American Society for Reproductive Medicine.)

Key Words: Ovarian cycle, immune response, type-1 cytokines, type-2 cytokines, progesterone, 17β-estradiol

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Type-1 and type-2 cytokine production was originally described among helper lymphocytes (CD4+ T lymphocytes; Th cells) in mouse (1) and later in humans (2). Mouse Th1 cells produce interleukin-2 (IL-2) and interferon-gamma (IFN-γ), whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Human Th1 and Th2 cells produce similar patterns, but the synthesis of cytokines is not as tightly restricted to a single subset as in mouse T cells (2). In addition, cytotoxic lymphocytes (CD8+ T cells; Tc cells) secrete a "Th1/Th2-like" cytokine pattern (3). The functions of Th1 and Th2 cells correlate well with their cytokine patterns: Th1 cells are involved in cellular immune responses, whereas Th2 cells are involved in humoral immune responses (4).

Th1 and Th2 cytokine patterns have now been implicated in various immune responses concerning infection, allergy, and autoimmunity (3). Pregnancy is also associated with alterations in Th1/Th2 balance; pregnancy appears to be a "Th2-type phenomenon" (5). The predominance of Th2-type cytokines in pregnancy could be important in avoiding rejection of the semiallogenic fetoplacental unit; for instance, IL-10 is protective for the fetus (6), whereas inflammatory responses and Th1 cytokines such as IL-2 and IFN are harmful for pregnancy (7). The trigger for modulation of

the maternal immune response is suggested to be the production of Th2 cytokines from the fetoplacental unit, diverting the maternal immune system away from damaging Th1-type immune responses (5).

It has, however, also been known for a long time that sex hormones may influence the immune response (8, 9) and, more recently, the influence of P and E_2 upon the production of Th1 and Th2 cytokines has been described (10, 11). It appears that in vitro, P increased the production of Th2 cytokines (10), whereas E2 increased IL-10 and IFN-γ production (11). Increased levels of these two hormones during pregnancy may therefore be able to induce the systemic shift toward a Th2-type response. This suggestion was tested in the present study with the use of the luteal phase of the normal ovarian cycle of healthy women as a state of increased P and 17β-E_2 concentrations. We compared this phase of the ovarian cycle with the follicular phase. Blood samples were taken from women in the follicular phase and the luteal phase, and lymphocytes were stimulated. Using flow cytometry, we measured the intracellular production of IFN-γ, IL-2, IL-4, and IL-10 in helper and cytotoxic lymphocytes.

MATERIALS AND METHODS

Reagents for Cell Activation and Cell Staining

We used the following reagents: monensin (Sigma, St. Louis, MO), phorbol myristate acetate (PMA; Sigma), FACS^{TM} lysing solution (Becton Dickinson Immunocytometry Systems; San Jose, CA), FACS^{TM} permeabilizing solution (Becton Dickinson), calcium ionophore A-23187 (Sigma), complete RPMI 1640 medium (GIBCO BRL, Breda, The Netherlands) supplemented with 60 μg/mL gentamicin, washing buffer (phosphate-buffered saline [PBS] with 0.5% bovine serum albumin and 0.1% NaN_3), and fixation buffer (0.5% paraformaldehyde in PBS).

Subjects

After obtaining Institutional Review Board approval and signed informed consent, we studied 13 healthy women with a menstrual cycle length between 26 and 32 days. Exclusion criteria were evidence of treatment with antibiotics or flulike symptoms within 14 days of the first blood sample, as well as the presence of any known diseases.

All blood samples (20 mL) were obtained in two Vacutainer^{TM} tubes (Becton Dickinson Vacutainer Systems, UK), one tube containing sodium heparin and the other tube containing ethylenediaminetetraacetic acid (EDTA). The first sample was obtained in the follicular phase of the ovarian cycle, 6–9 days after the first menstruation day; the second sample was taken in the luteal phase of the ovarian cycle, 6–9 days after a positive urine LH test (Clindia Benelux b.v., Leusden, The Netherlands).

Heparinized blood was used to evaluate intracellular cytokine production. EDTA blood was centrifuged at 3,000

rpm, and the plasma was frozen at $-20°C$ until later hormone analysis for 17β-E_2 and P according to the methods of Jurjens et al. (12) and de Jong et al. (13), respectively. Total white blood cell (WBC) count was determined with a microcell counter (Model Sysmex F800; Toa Medical Electronics Co., Ltd, Kobe, Japan).

Sample Processing

Antibodies

The following monoclonal antibodies were used (and, unless stated otherwise, all were purchased from IQ Products, Groningen, The Netherlands): Cy-Q–labeled mouse anti-human CD3 (clone B-B11), fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD8 (clone MCD8), FITC-labeled mouse anti-human CD14 (clone UCHM1), phycoerythrin-labeled mouse anti-human IFN-γ (clone 45-15), phycoerythrin-labeled mouse anti-human IL-2 (clone N7-48A), phycoerythrin-labeled mouse anti-human IL-4 (clone 8F-12), phycoerythrin-labeled mouse anti-human IL-10 (clone B-N10), phycoerythrin-labeled mouse isotype control IgG1 (clone MCG1), and FITC-labeled mouse anti-human CD94 (clone HP-3B1; Pharmingen, San Diego, CA).

Incubation

Immediately after sampling, 1 mL of heparinized whole blood was mixed with 1 mL of RPMI and stimulated with PMA (40 nM) and calcium ionophore (2 nM) for 4 hours at 37°C and 5% CO_2. One milliliter of heparinized blood was used as an unstimulated control and was mixed only with 1 mL of RPMI. In both the stimulated and unstimulated samples, monensin (3 μM) was added to enable accumulation of the cytokines in the Golgi complex by interrupting intracellular transport processes.

Sample Labeling

After stimulation, both the stimulated and unstimulated samples were aliquoted (0.2 mL per tube), and 1 mL of lysing buffer was added to all tubes. After 5 minutes of incubation at room temperature in the dark, all tubes were centrifuged and aspirated. The remaining pellets were resuspended in 0.5 mL of permeabilization buffer and incubated at room temperature in the dark for 10 minutes.

The cells were then washed once with ice-cold washing buffer. After aspiration, stimulated and unstimulated aliquots were incubated at room temperature in the dark with anti-CD3 (5 μL), anti-CD8 (5 μL), and either anti–IFN-γ, anti–IL-2, anti–IL-4, anti–IL-10, or isotype control IgG1 (5 μL) at saturating dilutions. For differential cell counts, two extra unstimulated aliquots were incubated with either anti-CD14 or anti-CD3/anti-CD94. After washing with washing buffer, the cells were fixed with ice-cold 0.5% paraformaldehyde in PBS and kept at 4°C in the dark until measured (within 24 hours).

Total WBC count and differential counts in the follicular and luteal phases of the ovarian cycle.

Varaible	Follicular phase		Luteal phase	
	Total no. of cells (10^7/L)	Percent of WBCs/ lymphocytes	Total no. of cells (10^7/L)	Percent of WBCs/ lymphocytes
WBCs	548 ± 27	—	680 ± 50[a]	—
Granulocytes	341 ± 19	62.6 ± 2.9	414 ± 41[a]	60.8 ± 2.9
Monocytes	24 ± 4	4.4 ± 0.7	31 ± 5[a]	4.7 ± 0.8
Lymphocytes	183 ± 18	33 ± 2.6	233 ± 23[a]	34.5 ± 2.5
T lymphocytes	134 ± 15	72.4 ± 2.2	168 ± 19[a]	71.7 ± 2.6
T-helper cells	84 ± 10	45.1 ± 2.6	104 ± 13[a]	44.2 ± 3.1
T-cytotoxic/suppressor cells	41 ± 5	22.5 ± 1.6	53 ± 7[a]	22.7 ± 1.7
NK cells	22 ± 3	12.6 ± 1.6	30 ± 5[a]	12.8 ± 2.0
T-helper/T-cytotoxic/suppressor cell ratio	2.15 ± 0.25	—	2.13 ± 0.31	—

Note: Values are means ± SEM.
[a] Statistically significant difference compared with the follicular phase (Wilcoxon, $P<.05$).

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Flow Cytometry

Cells were analyzed with the Coulter Epics Elite flow cytometer (argon-ion 488-nm laser) (Beckman-Coulter, UK). Two thousand lymphocytes were acquired while life gating on lymphocytes using CD3-positive cell-signal data were saved for later analysis. Analysis was performed using Win-list32 (Verity Software House, Inc., Topsham, ME).

Intracellular Cytokines

During analysis, a gate was set on lymphocytes using forward and side-scatter characteristics. Secondary gates were set on CD3+/CD8− (Th) cells and CD3+/CD8+ (Tc) cells. For both Th and Tc cells, single-parameter fluorescence histograms were defined for evaluation of intracellular cytokine production. Using the unstimulated control sample, linear gates were set in the histograms so that ≥99% of the unstimulated cells were negative for cytokine production. Results are expressed as the percentage of positive cells in the stimulated blood sample.

Differential Blood Cell Counts

Using forward and side-scatter characteristics, a gate was set on the total leukocyte population. Using forward and side-scatter characteristics as well as specific antibodies (CD3 for lymphocytes and CD14 for monocytes), we defined lymphocyte, monocyte, and granulocyte populations in an unstimulated aliquot of each sample and evaluated the percentages of lymphocytes, monocytes, and granulocytes in the total leukocyte population. Within the lymphocyte population, the percentages of CD3+, CD3+/CD8+, CD3+/CD8−, and CD3−/CD94+ (natural killer [NK]) cells were also evaluated.

Statistics

Results are expressed as mean ± SEM. To evaluate differences between the follicular and luteal phases, we used Wilcoxon's test. Differences were considered statistically significant at $P<.05$.

RESULTS

The mean age of our experimental subjects was 30.7 years (range, 27–34 years). Their mean cycle length was 27.1 days (range, 25–28 days).
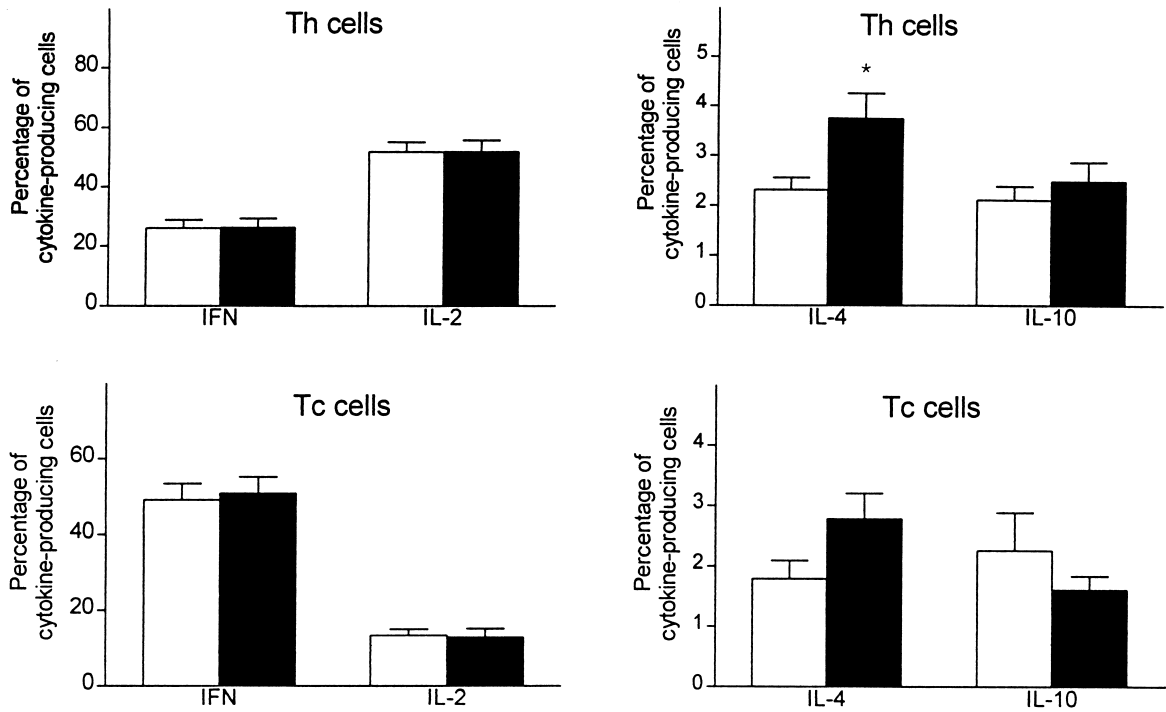
Plasma Hormone Concentrations

Both mean plasma 17β-E$_2$ and P concentrations were significantly increased in the luteal phase as compared with the follicular phase. Values for 17β-E$_2$ were: follicular phase, 0.39 ± 0.06 nM; luteal phase, 0.69 ± 0.03 nM (Wilcoxon, $P<.05$). For P, the follicular phase was 0.55 ± 0.16 nM and the luteal phase was 39.37 ± 4.3 nM (Wilcoxon, $P<.05$).

WBC Counts

Table 1 shows the mean total leukocyte counts in the follicular and luteal phases of the ovarian cycle of our experimental subjects as well as the percentages of granulocytes, monocytes, and lymphocytes and the percentages of lymphocytes that were helper, cytotoxic lymphocytes, or NK cells. The total leukocyte count was increased in the luteal phase of the ovarian cycle as compared with the follicular phase (Wilcoxon, $P<.05$). This increase was due to increases in granulocytes, monocytes, and lymphocytes (Wilcoxon, $P<.05$). The increase in lymphocytes consisted of increases in helper and cytotoxic lymphocytes as well as increases in NK cells (Wilcoxon, $P<.05$). The percentages of the various cell populations in the total WBC count did

Percentages of cytokine-producing cells (IFN-γ, IL-2, IL-4, and IL-10) of helper T-lymphocytes (top) and of cytotoxic T-lymphocytes (bottom) in the follicular phase and luteal phase of the normal ovarian cycle. $\square$ = follicular phase; $\blacksquare$ = luteal phase. *Statistically significant difference compared with the follicular phase (Wilcoxon, $P<.05$).

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not differ between the luteal phase and the follicular phase of the ovarian cycle.

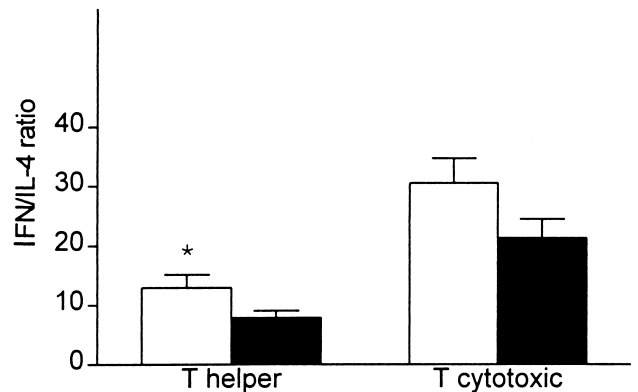## Intracellular Cytokine Production

Figure 1 shows the mean percentages of cytokine-producing cells for Th cells and Tc cells. The data show that the production of type-1 cytokines (IFN-γ and IL-2) and the type-2 cytokine IL-10 for both Th and Tc cells did not vary with the ovarian cycle. On the other hand, the production of the other type-2 cytokine studied, IL-4, was significantly increased in Th cells in the luteal phase as compared with the follicular phase of the ovarian cycle (paired t-test, $P<.05$). Figure 2 shows the IFN-γ to IL-4 ratio for Th and Tc cells. In Th cells, the IFN-γ to IL-4 ratio was significantly decreased in the luteal phase.

DISCUSSION

In the present study, we measured intracellular cytokine production by in vitro–activated helper and cytotoxic lymphocytes in a whole-blood preparation in the follicular and luteal phases of the ovarian cycle. Flow cytometry was used for analysis so that the production of (intracellular) cytokines could be measured at the single-cell level. The results show

Ratio of IFN-γ/IL-4–producing helper and cytotoxic T lymphocytes in the follicular phase and luteal phase of the normal ovarian cycle. $\square$ = follicular phase; $\blacksquare$ = luteal phase. *Statistically significant difference compared with the luteal phase (Wilcoxon, $P<.05$).

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that in the midluteal phase, as compared with the midfollicular phase, not only plasma P and 17β-E_2, but also the percentage of IL-4–producing cells after in vitro activation of Th cells were significantly increased. The percentages Th cells producing IL-10, IFN-γ, and IL-2 after in vitro activation did not differ between the follicular and the luteal phases of the ovarian cycle. In the cytotoxic T-cell population, no changes in cytokine production were observed between the follicular and the luteal phases of the ovarian cycle. The increased IL-4 production by Th cells results in a significantly decreased Th1/Th2 ratio during the luteal phase of the ovarian cycle.

In contrast to the suggestion of Wegmann et al. (5), our data demonstrate that for the shift in the Th1/Th2 ratio to occur, the fetoplacental unit need not be present. The fact that IL-4 production in Th cells changed in the ovarian cycle and the fact that hormone levels (i.e., P and 17β-E_2) changed in a similar pattern may support the concept that this shift is brought about by changes in the endocrine milieu. This view agrees with in vitro studies: P was found to increase IL-4 and IL-5 production after activation of established Th1 clones (10); the fact IFN-γ, IL-2, and IL-10 production of (helper and cytotoxic) lymphocytes did not vary with the phase of the menstrual cycle may suggest that these cytokines in vivo are not under regulatory control of the endocrine milieu. However, this is not in line with other in vitro experiments suggesting that E_2 increased IFN-γ production in activated T-helper cells (11). Although no studies have been done to show an effect of combined P and 17β-E_2 treatment upon cytokine production in T cells, it is feasible that in the luteal phase of the ovarian cycle, and possibly also during pregnancy, the increased concentrations of these hormones act in concert to establish a shift toward a Th2-type immune response.

Like the deviation toward a type-2 immune response, the total WBC count in the luteal phase was also changed in a "pregnancy-like" way. The WBC count was significantly increased in the luteal phase as compared with the follicular phase; this finding agrees with earlier observations (14, 15). The increase in total WBC counts in the luteal phase is due to increases in all subpopulations (granulocytes, monocytes, and lymphocytes) because the percentages of the subpopulations did not differ between the follicular and the luteal phases. Increases in granulocyte and monocyte numbers in the luteal phase have been reported before (14, 15) and are suggested to be due to increased 17β-E_2 concentrations, promoting the release of granulocytes and monocytes from the bone marrow (15). The increase in the lymphocyte population is also in line with previous studies (14) and, in the present study, was due to increased numbers of helper and cytotoxic T cells as well as NK cells.

The present results show that during the luteal phase of the normal ovarian cycle, the immune response shifts toward a type-2 response. This finding may have clinical implications. For example, during the luteal phase of the ovarian cycle (as in pregnancy), women with rheumatoid arthritis, a cell-mediated autoimmune disorder, often experience improvement of their symptoms (e.g., morning stiffness and pain) (16–18). The reason may be that both the luteal phase and pregnancy are associated with a type-2 immune response, shifting immunity away from the cell-mediated immune response. Moreover, diseases mediated by excessive autoantibody production (i.e., mediated by a Th2-type response), such as systemic lupus erythematosus, tend to flare up during pregnancy (19) and before menstruation (20), i.e., in the luteal phase of the ovarian cycle.

Although an effect of ovarian hormones upon the immune system has long been known (9), to our knowledge this is the first study to show that the response of the immune system to activation in vitro differs between the follicular and luteal phases of the ovarian cycle. The present results, together with previous results from our laboratory, showing a pregnancy-like inflammatory response in the luteal phase (21), strongly suggest that pregnancy-associated changes in the immune response are already observed in this phase of the normal ovarian cycle. The physiologic meaning of this phenomenon may be preparation of the maternal immune system for potential implantation of the semiallogenic blastocyst.

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