

The immune response during the luteal phase of the ovarian cycle: increasing sensitivity of human monocytes to endotoxin

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Objective: To test the hypothesis that during the luteal phase of the human ovarian cycle, as compared with the follicular phase, the percentage of cytokines producing peripheral monocytes after *in vitro* stimulation with endotoxin is increased.

Design: Prospective study.

Setting: Academic research institution.

Patient(s): Women with regular menstrual cycles.

Intervention(s): Blood samples were collected between days 6 and 9 of the menstrual cycle (follicular phase) and between days 6 and 9 of the menstrual cycle following the LH surge (luteal phase).

Main Outcome Measure(s): Percentages of tumor necrosis factor (TNF)- α -, interleukin (IL)-1 β -, and IL-12-producing monocytes as well as total white blood cell (WBC) count, differential WBC counts, and plasma 17 β -estradiol and progesterone concentrations.

Result(s): Mean plasma 17 β -estradiol and progesterone concentrations, percentage of TNF- α - and IL-1 β -producing monocytes, WBC counts, and granulocyte cell count were significantly increased in the luteal phase as compared with the follicular phase of the ovarian cycle. The percentage of IL-12-producing monocytes, monocyte count and lymphocyte count did not vary between the 2 phases of the ovarian cycle.

Conclusion(s): Together with an increase in progesterone and 17 β -estradiol during the luteal phase, there is an increase in percentage TNF- α - and IL-1 β -producing peripheral monocytes after *in vitro* stimulation with endotoxin as compared with the follicular phase of the ovarian cycle. Whether this increased sensitivity of monocytes for proinflammatory stimuli during the luteal phase is due to increased plasma levels of progesterone or 17 β -estradiol needs further investigation. (Fertil Steril® 2001;76:555-9. ©2001 by American Society for Reproductive Medicine.)

Key Words: Monocytes, cytokines, ovarian cycle, endotoxin, IL-12, IL-1 β , TNF- α

The interaction between the immune and reproductive systems has been a matter of interest for a long time, although the precise correlation between sex hormone levels and immune function has not been identified. In previous research with rats, we studied the effect of the reproductive condition on the response of the nonspecific immune system (inflammatory response). After infusion with low-dose endotoxin, the inflammatory response in pregnant rats is much more intense and persistent than in rats in the follicular phase of the ovarian cycle (1). It was demonstrated that this persistent inflammatory reaction is not only seen in pregnancy, but also exists in rats with an induced luteal phase (pseudopregnancy) (2).

These results suggest that hormonal factors, like progesterone or 17 β -estradiol, may be involved in the regulation of the endotoxin-induced inflammatory response in rats.

These results also suggest that such a situation could exist in humans as well. Recently, we demonstrated that the specific immune system changes during the luteal phase of the normal ovarian cycle, i.e., the immune response shifts toward a type-2 response (3). It was suggested that this deviation is hormonally determined and that physiologically this phenomenon might be the preparation of the maternal immune system for potential implantation of the semiallogenic blastocyst.

The present experiment was designed to

Received December 18, 2000; revised and accepted March 28, 2001.

Supported by the J.K. de Cock foundation (grant no. 00-12) and by an unrestricted educational grant by Organon.

Presented at the Second International Conference on Experimental and Clinical Reproductive Immunobiology, Amsterdam, The Netherlands, November 15-18, 2000.

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0015-0282/01/\$20.00
PII S0015-0282(01)01971-9

study the effect of the reproductive condition on the nonspecific immune response (inflammatory response) in humans. Monocytes are the first cellular component of the nonspecific immune system to be activated by endotoxin (lipopolysaccharide [LPS]) to produce various cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and IL-12 (4). Therefore, cytokine production of monocytes after *in vitro* endotoxin stimulation was used as a parameter for the state of activity and sensitivity of the nonspecific immune response. To study potential differences in response to endotoxin between the follicular and the luteal phase, we stimulated monocytes *in vitro* with endotoxin. The intracellular productive capacity of the peripheral blood monocytes TNF- α , IL-1 β , and IL-12 was measured by flow cytometry.

MATERIAL AND METHODS

Reagents for Cell Activation and Cell Staining

We used the following reagents: monensin (Sigma, St. Louis, MO), FACS™ lysing solution (Becton Dickinson Immunocytometry Systems, San Jose, CA), FACS™ permeabilizing solution (Becton Dickinson), complete RPMI 1640 medium (GIBCO BRL, Breda, The Netherlands) supplemented with 60 $\mu\text{g}/\text{mL}$ gentamycin, washing buffer (phosphate-buffered saline [PBS] with 0.5% bovine serum albumin and 0.1% NaN_3), and freezing buffer (10% DMSO in phosphate-buffered saline).

Subjects

After obtaining institutional review board approval and informed consent, 15 healthy women with a menstrual period length between 26 and 32 days were studied in the follicular and the luteal phase of a normal ovarian cycle. Exclusion criteria were evidence of treatment with antibiotics or flu-like symptoms (like fever, coughing, diarrhea, a cold, or vomiting) within 14 days of the first blood sample or during the sampling cycle, as well as the presence of any known diseases or any use of medication.

All blood samples (20 mL) were obtained in two vacutainer tubes, one tube containing sodium heparin, the other ethylenediaminetetraacetate (EDTA). The first sample was obtained in the follicular phase of the ovarian cycle, i.e., 6–9 days after the first day of menstruation; the second sample in the luteal phase of the ovarian cycle, i.e., 6–9 days after a positive urinary LH test (Clindia; Benelux b.v., Leusden, The Netherlands). The EDTA blood sample was used for total white blood cell counts using a microcellcounter (Model Sysmex F800; Toa Medical Electronics Co., Kobe, Japan). EDTA plasma was subsequently isolated after centrifugation of the sample at 3,000 rpm. The plasma was frozen at -20°C until later hormone analysis [17β -estradiol and progesterone according to the method of Jurjens et al. (5) and de Jong et al. (6), respectively]. Heparinized blood was used to evaluate intracellular cytokine productive capacity.

Sample Processing

Antibodies

The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD14 (clone UCHM1; IQ Products, Groningen, The Netherlands); phycoerythrin (PE)-labeled mouse anti-human TNF α (clone Mab11; Pharmingen); PE-labeled mouse anti-human IL-1 β (clone AS10; Becton Dickinson) and PE-labeled mouse anti-human IL-12 (clone C11.5; Pharmingen); and PE-labeled mouse isotype control IgG1 (clone MCG1; IQ Products).

Sample Incubation

Immediately after sampling, 1 mL of heparinized whole blood was mixed with RPMI and stimulated with 2 $\mu\text{g}/\text{mL}$ of LPS (*E. coli*, 0.55:B5, Whittaker; stimulated sample). One milliliter of heparinized blood was used as unstimulated control and only mixed 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. Stimulated and unstimulated samples were incubated for 4 hours at 37°C and 5% CO_2 .

Sample Labeling

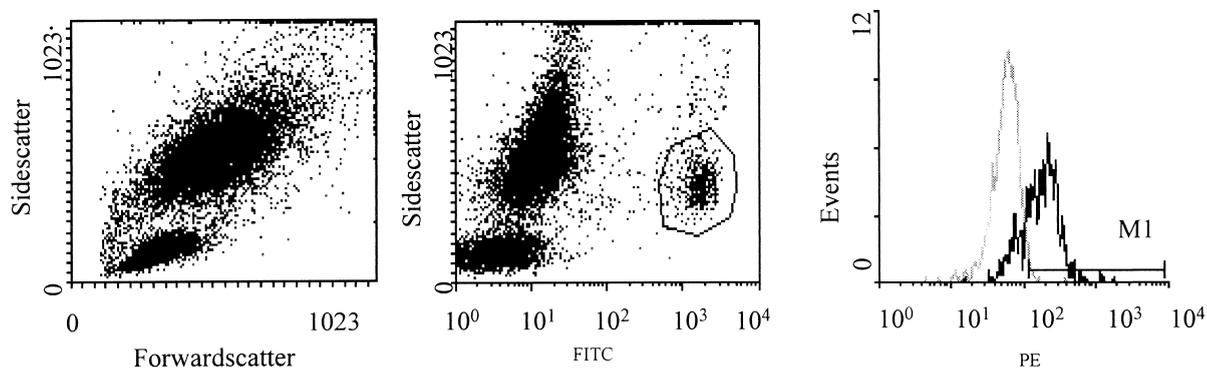
After incubation, both stimulated and unstimulated samples were aliquoted (0.2 mL per tube) and 5 μL of anti-CD14 was added to each tube. Tubes were incubated at RT in the dark for 30 minutes. Following incubation with 1 mL of lysing buffer for 5 minutes in the dark, tubes were centrifuged and aspirated. Cells were then washed with 2 mL of washing buffer, after which 0.2 mL of freezing buffer was added to each tube. Tubes were frozen at -80°C . All tubes (from follicular and luteal phase) from one subject were thawed at the same day. After thawing and two washes, the remaining pellets were resuspended in 0.5 mL of permeabilizing buffer and incubated in the dark for 10 minutes. Then the cells were washed with ice-cold washing buffer. After centrifugation and aspiration, stimulated and unstimulated aliquots were incubated for 30 minutes in the dark at RT with either anti-TNF α , anti-IL-1 β , anti-IL-12, or isotype control (5 μL) at saturating dilutions. After washing with washing buffer, cells were fixed with fixation buffer and kept at 4°C in the dark until measured (within 24 h).

Flow Cytometry

Cells were analyzed using the Coulter Epcis flow cytometer (Argon-ion 488 nm laser). One thousand monocytes were acquired while life-gating on monocytes using the CD14+ cell signal and saved for later analysis. Analysis was performed using Winlist32 (Verity Software House, Inc., Topsham, ME).

FIGURE 1

IL-1 β production of CD14 $^{+}$ monocytes. After whole blood stimulation with endotoxin (lipopolysaccharide), granulocytes and lymphocytes can be demonstrated separately in a forward scatter (FSC) and sideward scatter (SSC) dotplot (left dotplot). To analyze cytokine production by monocytes (CD14 $^{+}$ cells), monocytes were first selected in a region R1 on an SSC and CD14mAb-fluorescein isothiocyanate dotplot (middle dotplot). A single-parameter fluorescence histogram was then used to evaluate the number of PE-positive cells in this monocyte R1 region. Using the unstimulated control sample, linear gates were set in the histogram (M1) so that at least 99% of the unstimulated cells were negative for cytokine production. This gate was then copied to the histogram of the stimulated cells; results are expressed as percentage of positive cells in the stimulated blood sample (right histogram: gray line, unstimulated sample; black line, stimulated sample).



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Data Analysis of Intracellular Cytokines

During analysis, a gate was set on CD14 $^{+}$ monocytes. A single-parameter fluorescence histogram for the monocytes was defined for evaluation of intracellular cytokine production. For evaluation of cytokine production following *in vitro* LPS stimulation, unstimulated and stimulated aliquots were used. Using the unstimulated aliquot, a linear gate was set in the histogram so that at least 99% of the cells in this aliquot were negative for cytokine production (Fig. 1). This gate was then copied to the histogram of the stimulated cells; results are expressed as percentage of positive cells in the stimulated aliquots.

Differential Blood Cell Counts

Total white blood cell (WBC) number was counted on a microcellcounter (Model Sysmex F800; Toa Medical Electronics Co.). Differential WBC counts were made using flow cytometry data; by taking advantage of the differences in sidescatter characteristics between lymphocytes and monocytes/neutrophils as well as the different CD14 expression of monocytes and neutrophils, the percentages of different cell populations were counted.

Statistics

Results are expressed as mean percentage of positive monocytes \pm SEM. To evaluate differences between the follicular and luteal phase, Wilcoxon's signed rank test was used. Differences are considered to be significant if $P < .05$.

RESULTS

The mean age of the women in this study was 30.7 years (range, 27–36 years). Their mean cycle length was 27.9 days (range, 26–32 days). Blood samples were taken at day 6.9 (range, 6–8 days) of the follicular phase and at 6.1 days after a positive urinary LH surge (range, 6–8 days) in the luteal phase.

Plasma Concentrations

The 17 β -estradiol and progesterone concentrations were significantly increased in the luteal phase as compared with the follicular phase (17 β -estradiol: follicular phase, 0.27 ± 0.03 nM; luteal phase, 0.56 ± 0.07 nM [Wilcoxon, $P < .05$]. Progesterone: follicular phase, 1.03 ± 0.21 nM; luteal phase, 50.49 ± 7.23 nM [Wilcoxon, $P < .05$]).

WBC Counts

Table 1 shows the mean total leukocyte counts in the follicular and luteal phases of the ovarian cycle as well as the percentages of granulocytes, monocytes, and lymphocytes. There were no significant differences in total number of monocytes and lymphocytes in the luteal phase as compared with the follicular phase. However, WBC count and the granulocyte count were significantly increased in the luteal phase of the ovarian cycle as compared with the follicular phase (Wilcoxon, $P < .05$). The percentages of the various cell populations in the total WBC count did not differ between the two phases of the ovarian cycle.

TABLE 1

Total white blood cell (WBC) count and differential counts in the follicular and luteal phase of the ovarian cycle.

Variable	Follicular phase, total no. of cells (10 ⁷ /L)	Percentage of WBCs	Luteal phase, total no. of cells (10 ⁷ /L)	Percentage of WBCs
WBCs	553 ± 28	—	639 ± 35 ^a	—
Granulocytes	328 ± 27	58.9 ± 2.3	383 ± 32 ^a	60.0 ± 2.9
Monocytes	27 ± 3	5.0 ± 0.5	33 ± 4	5.5 ± 0.6
Lymphocytes	198 ± 12	36.1 ± 2.4	223 ± 23	34.5 ± 3.0

Note: Values are mean ± SEM.

^a Statistically significant difference vs. the follicular phase (Wilcoxon, *P* < .05).

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Intracellular Cytokine Production

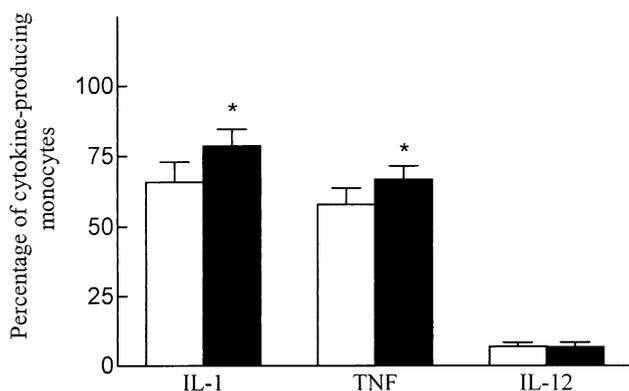
Figure 2 shows the percentages of IL-12-, TNF- α -, and IL-1 β -producing monocytes in the two phases of the ovarian cycle after in vitro stimulation with endotoxin. As can be seen from this figure, the percentage of IL-12-producing monocytes does not vary between the two phases of the ovarian cycle. However, the percentage of IL-1 β - and TNF- α -producing monocytes is significantly increased in the luteal phase as compared with the follicular phase (Wilcoxon's signed rank test, *P* < .05).

DISCUSSION

We found a significant increase in both the percentage of IL-1 β -producing monocytes and percentage of TNF- α -producing monocytes in the luteal phase as compared with the follicular phase. However, we found no difference in the percentage of IL-12-producing monocytes.

FIGURE 2

Percentage of cytokines producing (IL-1 β , TNF- α , and IL-12) monocytes in the follicular phase (open bars) and the luteal phase (closed bars) of the normal ovarian cycle.



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Other studies already demonstrated a relationship between reproductive condition and the nonspecific immune system response. Cannon et al. described an increase in plasma IL- β concentrations in the luteal phase as compared with the follicular phase during the normal ovarian cycle (7), while Brännström described a significant increase in plasma TNF- α level during the late follicular and during the mid luteal phase as compared with the early follicular phase of the normal ovarian cycle (8). Polan found a 3-fold increase in IL-1 β mRNA in peripheral monocytes during the luteal phase over that found in the follicular phase (9). All the above mentioned studies were performed with resting peripheral blood mononuclear cells, i.e., without in vitro stimulation with endotoxin, and demonstrated a higher production of mRNA and a higher correlating bioactive peptide secretion of IL-1 β and TNF- α in the luteal phase as compared with the follicular phase of the ovarian cycle in humans.

To our knowledge this is the first study showing that the response of the monocytes to activation by in vitro stimulation with endotoxin differs between the follicular and the luteal phase of the ovarian cycle, indicating that monocytes are more sensitive to proinflammatory stimuli (endotoxin) in the luteal phase as compared with the follicular phase. Therefore, we conclude that monocytes not only produce higher amounts of TNF- α and IL-1 β (7–9) but also appear to be in a higher state of excitation in the luteal phase.

Because progesterone and 17 β -estradiol plasma concentrations increase concomitantly with the increase in sensitivity of monocytes to proinflammatory stimuli in the luteal phase, it may be suggested that sex hormones can modulate the sensitivity of monocytes to proinflammatory stimuli. In vitro studies have been performed to define the relationship between monocyte cytokine production and sex hormones. Polan et al. demonstrated that endotoxin-stimulated human peripheral blood monocyte levels of both TNF- α mRNA and IL-1 β mRNA increased when incubated with physiologic levels of progesterone and 17 β -estradiol concentrations (10, 11). Also, without concomitant endotoxin stimulation, hu-

man monocyte IL-1 activity was increased by physiologic levels of sex hormones (12). However, it remains elusive whether these results are caused by a direct effect of progesterone or estradiol on monocytes, because as yet we have found no evidence of the presence of estrogen or progesterone receptors on monocytes.

Maybe there are factors other than these hormones responsible for modulating the activity and sensitivity of monocytes during the ovarian cycle. It may even be possible that monocytes are not activated or are more sensitive in the luteal phase and that monocytes are suppressed and less sensitive in the follicular phase. We have previously demonstrated that in pregnant rats part of the endotoxin-induced glomerular inflammatory response can be inhibited by induction of follicular growth during pregnancy. It was therefore suggested that a follicular ovarian factor exists, capable of suppressing the nonspecific immune system during the follicular phase (13, 14). Once this factor disappears (in the luteal phase or during pregnancy), the nonspecific immune system is not suppressed anymore and thus appears to be "activated and more sensitive."

In our study we investigated monocytes as a parameter for the nonspecific immune response. The current observations concerning an "increase in activity and sensitivity" of the nonspecific immune response together with a previously demonstrated deviation of a Th2 type response of the specific immune response in the luteal phase of the ovarian cycle (3) not only raises the question of what factors are involved in the control of the immune response, but also raises the question of why the immune response varies so strongly with the reproductive condition. A possible answer might be that the physiologic meaning of this phenomenon may be the preparation of the maternal immune system for potential implantation of the semiallogenic blastocyst (3, 15).

In summary, we demonstrated an increase in IL-1 β - and TNF- α -producing monocytes after in vitro endotoxin stimulation in the luteal phase as compared with the follicular

phase of the ovarian cycle, concomitant with an increase in progesterone and 17 β -estradiol. Whether this increase is directly or indirectly due to the increase in plasma progesterone and plasma estradiol concentration, or to the absence of a follicular factor in the luteal phase, needs further investigation.

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