

Cytokine production in natural killer cells and lymphocytes in pregnant women compared with women in the follicular phase of the ovarian cycle

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Objective: To test whether peripheral natural killer (NK) cells, helper T cells, and cytotoxic lymphocytes of pregnant women shift from a type 1 cytokine production toward a type 2 cytokine production as compared with these cells in women in the follicular phase.

Design: Prospective study.

Setting: Outpatient clinic.

Patient(s): Healthy nullipara at 30 weeks' amenorrhea and healthy nonpregnant women in their follicular phase.

Intervention(s): Samples of whole blood were stimulated with phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, MO) and Ca-ionophore in the presence of monensin (Sigma). Lymphocytes were stained with α -CD3, α -CD8, and α -interferon gamma (IFN- γ) α -interleukin 2 (IL-2), IL-4, or IL-10. Analysis was performed by flow cytometry. Statistical evaluation was done with the Mann-Whitney *U* test.

Main Outcome Measure(s): Percentage NK cells, helper lymphocytes, and cytotoxic lymphocytes that were producing IFN- γ , IL-2, IL-4, or IL-10.

Result(s): There is a statistically significant decrease in the percentage of NK cells, and helper and cytotoxic lymphocytes that produced IFN- γ in pregnant women when compared with women in the follicular phase. There is also a statistically significant decrease in the percentage of helper lymphocytes producing IL-2 in pregnant women compared with nonpregnant women.

Conclusion(s): We found a decrease in type 1 cytokine production with no change in type 2 cytokine production after in vitro stimulation of "pregnant" NK cells and lymphocytes as compared with "nonpregnant" NK cells and lymphocytes. We suggest that NK cell and lymphocyte response are shifted away from a type 1 immune response during pregnancy. (Fertil Steril® 2002;77:1032-7. ©2002 by American Society for Reproductive Medicine.)

Key Words: Pregnancy, natural killer cells, lymphocytes, type 2 immune response, cytokines

Type 1 and type 2 cytokine production was originally described among helper lymphocytes (CD4⁺ T lymphocytes; Th cells) in mice (1) and later in humans (2). Mouse T_H1 cells produce interleukin-2 (IL-2) and interferon-gamma (IFN- γ), whereas T_H2 cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-13 (IL-13) (1). Human T_H1 and T_H2 cells produce similar patterns, but the synthesis of cytokines is not as tightly restricted to a single subset as in mouse Th cells (2). Also,

cytotoxic lymphocytes (CD8⁺ T cells; Tc cells) secrete type 1 or type 2 cytokines (3).

The cytokine repertoire of peripheral natural killer (NK) cells is mainly type 1 cytokines; however, it is reported that NK cells are also capable of producing type 2 cytokines (4-7). In vitro experiments have demonstrated that a differentiation in the type 1 and type 2 NK cells can be made that is comparable to the cytokine types in T helper lymphocytes (8).

It has been suggested that type 1 cytokines,

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which are involved in cellular immune responses, are harmful for maintenance of pregnancy, whereas type 2 cytokines, which are involved in humoral immune responses, appear to be protective for the fetus (9–13). Therefore, it seems likely that type 2 cytokines dominate over type 1 cytokines during pregnancy.

Previous studies, however, have shown conflicting results concerning the production of type 1 and type 2 cytokines of peripheral lymphocytes during pregnancy when pregnant women were compared with women who were not pregnant (14, 15). These conflicting results may be due to not selecting between the follicular and luteal phase in the nonpregnant women. In a previous study, we showed that a shift toward a type 2 immune response can already be observed during the luteal phase of the ovarian cycle when it is compared with the follicular phase (16).

Thus, the aim of our study was to compare the production of type 1 and type 2 cytokines by the peripheral Th and Tc cells of pregnant women with the production of these cytokines by lymphocytes of women in the follicular phase of the ovarian cycle. Moreover, our study also included peripheral NK cells and compared the production of type 1 and type 2 cytokines by these cells during pregnancy with production of cytokines in these cells during the follicular phase of the ovarian cycle. The NK cells and lymphocytes in whole blood of pregnant women and of women in the follicular phase of the ovarian cycle were stimulated, and the intracellular production of IFN- γ , IL-2, IL-4, and IL-10 was measured using flow cytometry.

MATERIAL AND METHODS

List of Reagents for Cell Activation and Cell Staining

The reagents used for cell activation and staining were Monensin (Sigma Chemical Co., St. Louis, MO), phorbol myristate acetate (PMA; Sigma), FACS lysing solution (Becton Dickinson, Heidelberg, Germany), FACS permeabilizing solution (Becton Dickinson), calcium ionophore A-23187 (Sigma); Complete RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 60 $\mu\text{g}/\text{mL}$ gentamycin; washing buffer (phosphate buffered saline with 0.5% bovine serum albumin and 0.1% NaN_3), and fixation buffer (0.5% paraformaldehyde in phosphate buffered saline).

Patients

After obtaining institutional approval from the local ethics committee, and after the participants had signed informed consent, we took blood samples from healthy nulliparous women ($n = 17$, age range: 18 to 35 years) at 30 weeks' amenorrhea (recruited from our outpatient clinic for midwives) and from healthy women who were not pregnant ($n = 12$, age range: 18 to 35 years) with a menstrual cycle length between 26 and 32 days (hospital staff and students). In this latter group, the blood samples were taken during the wom-

en's follicular phase, 6 to 9 days after the first day of menstruation. For both groups, the exclusion criteria were evidence of treatment with antibiotics or flu-like symptoms within 14 days of the blood sampling as well as the presence of any known diseases. Blood samples (20 mL) were obtained in two vacutainer tubes (Becton Dickinson): one tube contained sodium heparin and was used to evaluate intracellular cytokine production; the other tube contained EDTA and was used for white blood cell (WBC) counts (using a microcellcounter, Model Sysmex F800; Toa Medical Electronics, Kobe, Japan).

Sample Processing

Antibodies

The following monoclonal antibodies were used (unless stated otherwise, all antibodies were purchased from IQ Products in 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 (PE) labeled mouse anti-human IFN- γ (clone 45-15), PE labeled mouse-anti-human IL-2 (clone N7-48A), PE labeled mouse anti-human IL-4 (clone 8F-12), PE labeled mouse anti-human IL-10 (clone B-N10), PE labeled mouse isotype control IgG1 (clone MCG1), FITC labeled mouse anti-human CD94 (clone HP-3B1; Coulter Immunotech, Hamburg, Germany).

Incubation

Immediately following sampling, 3 mL of heparinized whole blood was mixed with 3 mL of RPMI and stimulated with PMA (40 nM) and calcium ionophore (2 nM) for 4 hours at 37°C and 5% CO_2 . The unstimulated control was 3 mL of heparinized whole blood, which was only mixed with 3 mL of RPMI. In both the stimulated and unstimulated sample, Monensin (3 μM) was added to enable accumulation of the cytokines in the golgicomplex by interrupting intracellular processes.

Sample Labeling

After stimulation, both samples were aliquoted (0.2 mL per tube) and 1 mL of lysing buffer was added to all tubes for lysing the red blood cells. 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 were incubated at room temperature in the dark for 10 minutes. Then the cells were washed once with ice-cold washing buffer. After centrifugation and aspiration, the stimulated and unstimulated aliquots were incubated at room temperature in the dark for 30 minutes 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). Other aliquots were incubated with anti-CD3 (5 μL), anti-CD94 (10 μL), and either anti-IFN- γ ,

TABLE 1

Numbers and percentages of cells in pregnancy and in the follicular phase of the ovarian cycle.

Variable	Follicular phase		Normal pregnancy	
	Total no. of cells (10 ⁷ cells/L)	White blood cells/lymphocytes (%)	Total no. of cells (10 ⁷ cells/L)	White blood cells/lymphocytes (%)
White blood cells	548 ± 27		1,104 ± 80 ^a	
Granulocytes	341 ± 19	62.6 ± 2.9	864 ± 65 ^a	79.0 ± 1.2 ^a
Monocytes	24 ± 4	4.4 ± 0.7	43 ± 5 ^a	3.7 ± 0.4
Lymphocytes	183 ± 18	33 ± 2.6	155 ± 16	16.9 ± 1.1 ^a
T lymphocytes	134 ± 15	72.4 ± 2.2	116 ± 13	71.7 ± 1.0
T helper cells	84 ± 10	45.1 ± 2.6	79.2 ± 8.1	51.2 ± 1.8
T cytotoxic cells	41 ± 5	22.5 ± 1.6	39.7 ± 8.3	23.6 ± 1.8
NK cells	22 ± 3	12.6 ± 1.6	9.8 ± 1.6 ^a	7.3 ± 0.8 ^a
T helper/T cytotoxic ratio	2.15 ± 0.25		2.4 ± 0.3	

^a = Statistically significant difference from follicular phase (Mann-Whitney *U* test, *P* < .05).

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anti-IL-2, anti-IL-4, anti-IL-10, or isotype control IgG1 (5 µL) at saturating dilutions. For differential cell counts, one extra unstimulated aliquot was incubated with anti-CD14. After they were washed with washing buffer, cells were fixed with ice-cold 0.5% paraformaldehyde in PBS and kept at 4°C in the dark until measurements were performed (within 24 hours).

Flow Cytometry

Cells were analyzed using the Coulter Epics Elite flow cytometer (Argon-ion 488-nm laser; Coulter, Luton, UK). For each individual, 2,000 lymphocytes were acquired by the use of life gating on lymphocytes using forward and side-scatter characteristics (to exclude doublets and triplets) and CD3⁺ cell signal. Data were saved for later analysis. To minimize any day-to-day variation of the measurements, the PE fluorescence (i.e., the label for the antibodies) was calibrated before each experiment using Standard Brite Beads (Coulter). Analysis was performed using WinList 3.2 (Verity Software House, 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) or CD3⁻/CD94⁺ (NK cells) cells. For Th, Tc, and NK 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 at least 99% of the unstimulated cells were negative for cytokine production. This gate was copied to the histogram for stimulated cells. Results are expressed as the percentage of positive cells in the stimulated blood sample. The T1/T2 ratio was calculated by dividing the

percentage of cells producing INF-γ by the percentage of cells producing IL-4.

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), the lymphocyte, monocyte, and granulocyte populations were defined in an unstimulated aliquot of each sample and the percentage of lymphocytes, monocytes, and granulocytes of the total leukocyte population were evaluated. Within the lymphocyte population, the percentage of CD3⁺, CD3⁺/CD8⁺, and CD3⁺/CD8⁻ and CD3⁻/CD94⁺ (NK cells) cells also were evaluated.

Statistics

The results are expressed as mean ± standard error of the mean (SEM). To evaluate differences between the follicular phase and pregnancy, the Mann-Whitney *U* test was used. A difference of *P* < .05 was considered statistically significant.

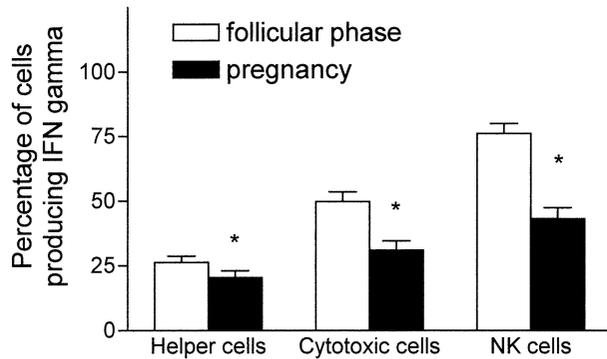
RESULTS

White Blood Cell Counts

Table 1 shows the total white blood cell counts and differential counts in the follicular phase of the ovarian cycle and in normal pregnancy. The total white blood cell count was significantly increased in pregnant women compared with that of the women in the follicular phase (Mann-Whitney, *P* < .05). This increase was due to an increase in the number of granulocytes and monocytes (Mann-Whitney, *P* < .05). Moreover, a decrease in NK cells was seen in pregnant women as compared with women in the follicular phase.

FIGURE 1

Percentage of cell producing INF- γ in pregnant women as compared with women in the follicular phase. (*Significantly different from follicular phase; Mann-Whitney *U* test, $P < .05$.)



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NK Cell Cytokines in Follicular Phase Compared With 30-Weeks' Amenorrhea Pregnancy

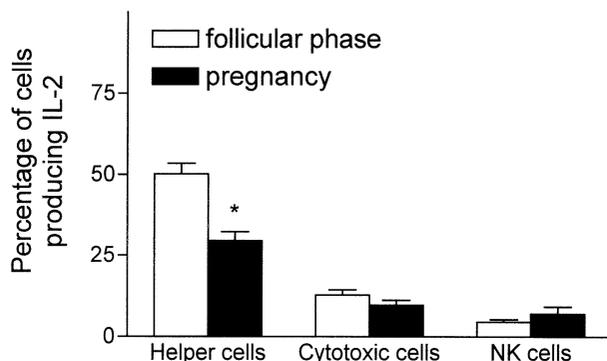
Comparing pregnancy with the follicular phase, the percentage of NK cells producing IFN- γ was significantly decreased. The other type 1 cytokine, IL-2, as well as type 2 cytokines remained stable when the two groups are compared (Figs. 1–4).

T Helper Cytokines in Follicular Phase Compared With 30-Weeks' Amenorrhea Pregnancy

In T helper cells a significant decrease in the percentage of cells producing the type 1 cytokines IFN- γ and IL-2 in

FIGURE 2

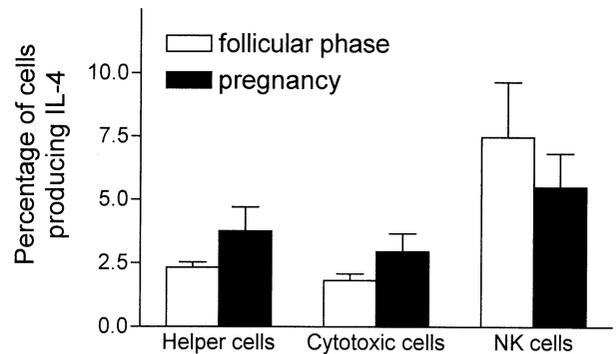
Percentage of cells producing IL-2 in pregnant women as compared with women in the follicular phase. (*Significantly different from follicular phase; Mann-Whitney *U* test, $P < .05$.)



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FIGURE 3

Percentage of cells producing IL-4 in pregnant women as compared with women in the follicular phase. (*Significantly different from follicular phase; Mann-Whitney *U* test, $P < .05$.)



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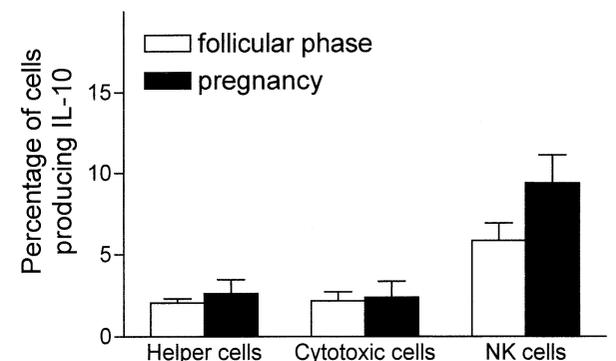
pregnancy as compared with the follicular phase was seen (Figs. 1 and 2). Comparing follicular phase with pregnancy, the percentage of cells producing type 2 cytokine remained stable.

T Cytotoxic Cytokines in Follicular Phase Compared With 30-Weeks' Amenorrhea Pregnancy

The percentage of T cytotoxic cells producing type 1 cytokine IFN- γ was significantly decreased in pregnancy when compared with the follicular phase of the ovarian cycle (Fig. 1). The percentage of cells producing IL-2 and the type 2 cytokines (IL-4 and IL-10) remained stable for both pregnancy and the follicular phase (Figs. 2–4).

FIGURE 4

Percentage of cells producing IL-10 in pregnant women as compared with women in the follicular phase. (*Significantly different from follicular phase; Mann-Whitney *U* test, $P < .05$.)



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The T1/T2 ratio in helper cells was 13.24 ± 1.91 in the follicular phase, which was significantly higher as compared with this ratio in pregnancy (10.07 ± 3.03). The T1/T2 ratio in cytotoxic cells was 33.87 ± 6.0 in the follicular phase, which was significantly higher when compared with the ratio for pregnant women (15.56 ± 2.98).

DISCUSSION

Our study used flow cytometry to show that, on in vitro stimulation, the cytokine response in peripheral NK cells and lymphocytes in pregnant women (as compared with women in the follicular phase) is shifted away from a type 2 response. We choose to compare pregnant women with follicular-phase nonpregnant women because we have shown previously that there is a shift toward a type 2 response in the luteal phase of the normal ovarian cycle as compared with the follicular phase (16).

The present method was chosen because, in contrast to measuring cytokine production in supernatants of stimulated lymphocytes, it allowed us to investigate cytokine production in NK cells, Th cells, and Tc cells separately. We also show, in line with several other studies, an increase in the number of white blood cells, granulocytes, and monocytes and a decrease in the number of NK cells in pregnant women when compared with women who are not pregnant. The T lymphocytes and T lymphocyte-subsets showed no differences between pregnant women and nonpregnant women (17, 18).

To our knowledge, this is the first study to evaluate the cytokine production of peripheral NK cells. The percentage of NK cells producing IFN- γ in pregnancy after in vitro activation was decreased compared with the follicular phase. The percentage of NK cells producing the other cytokines, IL-2, IL-4, IL-10, did not differ between follicular phase and third-trimester pregnancy.

Natural killer cells are very important in the cellular immune response. They also play an important role in decidualization (19). Weill (19) first appreciated the potential role of NK-like cells in the decidual response in pregnancy in 1921 and described the presence of endometrial stromal granulocytes in the decidua. These granulocytes seemed to be lymphocytes that have many similarities with NK cells (large granular lymphocytes [LGL]). Only few studies have been done to evaluate peripheral NK cell activity during human pregnancy: one study observed that NK cell activity appeared to decrease from 16 weeks until term, returning to control levels after delivery (20), whereas other investigators have shown a decrease in NK cell numbers during pregnancy with the remaining cells having less lytic activity (21).

The decrease in T lymphocytes (both Th and Tc) producing type 1 cytokine during pregnancy compared with the follicular phase is obvious, resulting in a decreased T1/T2 balance. These results are in agreement with a study of Saito

et al. (22), who used a similar experimental setup to our own study. They also found decreased numbers of cells producing IFN- γ after in vitro stimulation of lymphocytes of pregnant women as compared with lymphocytes of nonpregnant women and no difference in the lymphocytes producing IL-4.

Reinhard et al. (23) showed that the T_H1/T_H2 ratio in T cells during pregnancy was significantly decreased. However, in contrast to our study and that of Saito et al. (22), they found this to be due to a decreased production of T_H1 cytokines (IFN- γ and IL-2) as well as an increased production of IL-4 in normal pregnant women compared with nonpregnant women. The Reinhard study stimulated isolated peripheral blood mononuclear cells (PBMC) instead of whole blood. By the use of a different method of stimulating lymphocytes, Ekerfelt et al. (24) reported finding, during the second and third trimester of pregnancy, significantly increased numbers of PBMC secreting IL-4 but an unchanged number of cells secreting IFN- γ .

The different outcomes among the studies might possibly be explained by the use of different methods of stimulation or the different methods of measuring cytokine production. The differences of our study compared with other studies cannot be explained by the more restricted control group we used: IFN- γ production by lymphocytes and NK cells did not differ for women in the follicular phase compared with women in the luteal phase of the ovarian cycle, whereas IL-4 production of lymphocytes was decreased during the follicular phase. Our study showed that IL-4 production in this phase did not differ from IL-4 production during pregnancy. Thus, the outcome of our study would probably not have been different if instead we had used nonpregnant women without selection for the phase of the cycle.

Various mechanisms may account for the decrease in IFN- γ production by NK cells and lymphocytes during pregnancy.

First, the changes in hormone levels during pregnancy, such as increased progesterone, may be responsible for this decrease (25). However, after the stimulation of T_H1 clones incubated with progesterone, IL-4 and IL-5 production was increased as compared with the same clones that were not incubated with progesterone, whereas no effect was observed upon IFN- γ production (25). This may suggest that progesterone does not play a role in the shift in the decreased IFN- γ production found for pregnancy in our study. This suggestion is in line with a previous study from our laboratory: comparing the luteal phase (with increased progesterone) with the follicular phase, we found no difference in lymphocytes producing IFN- γ between these two phases (16). In addition, progesterone is not likely to play a role for NK cells in the decrease in IFN- γ , because no difference in percentage of NK cells producing IFN- γ has been observed between the follicular and the luteal phase of the normal ovarian cycle (6).

As progesterone is not likely to play a role in the inhibition of the percentage of lymphocytes and NK cells producing IFN- γ , the presence of the fetomaternal unit may be needed for the shift away from a type 1 response at 30 weeks of pregnancy (9). The exact mechanism by which the fetomaternal unit interferes in the immune response remains unknown. Because the maternal blood at the intervillous space is in close contact with the trophoblast cells, these cells may affect the maternal lymphocytes.

Indeed, trophoblast cells and maternal cells (i.e., decidual cells, leukocytes, and cells of the lymph nodes draining the uterus) appear to be able to suppress immune responses in vitro (26, 27). In addition, factors produced by these cells in culture have been proposed to serve immunomodulatory roles (28). Further insight into the roles of these maternal and fetal cells in the regulation of the immune response is needed for a clearer understanding of the working mechanism of the immune response in pregnancy (29).

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