CYCLIC PLASMA IL-6 LEVELS DURING NORMAL MENSTRUAL CYCLE

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Steroid hormones including sex hormones are known to influence cytokine production by cells in vitro. We investigated whether there are differences in cytokine production in vivo and ex vivo during the menstrual cycle in five ovulating women compared with five pregnant women and nine males. Interleukin 6 (IL-6) in plasma changed periodically during 12 of 13 cycles in five women. The IL-6 levels were lowest in the luteal phase when progesterone levels were elevated and highest preovulatory when progesterone levels were low ($P < 0.009$). This phenomenon was unrelated to changes in haematocrit or albumin and independent of cortisone, growth hormone, luteinizing or follicle stimulating hormone and testosterone. In contrast to IL-6, the soluble IL-6 receptor did not vary significantly during the menstrual cycle. In comparison, nine males and five pregnant women had low plasma IL-6 levels comparable with women during the luteal phase. In addition, levels of IL-6, IL-10 and TNF were determined after whole blood stimulation with lipopolysaccharide ex vivo during a menstrual cycle. Neither the number of CD-14^{++} or CD14/CD16^{+} cells nor the amounts of IL-6, IL-10 and TNF after stimulation showed cyclic changes.

We suggest that sex hormones, especially oestrogen and progesterone, may influence immune responses by decreasing basal IL-6 levels in vivo.

Most of the autoimmune diseases are more frequent in females than in males.[1] This may implicate that gender specific hormones may be involved in little-known mechanisms leading to the autoimmune reaction. The most dramatic changes in progesterone and oestrogen levels are found during pregnancy in which autoantibody titres as well as the activity of, i.e. Graves' disease or rheumatoid arthritis are decreased. After delivery these diseases are exacerbated. This implicates that, i.e. oestrogen may ameliorate the autoimmune process. However treatment with oestrogen had no positive effect in rheumatoid arthritis. In contrast, the systemic lupus erythematodes is known to be exacerbated under oestrogen therapy or pregnancy.

The immunomodulatory mechanisms induced by sex hormones (SH) are only partially understood. Most of the knowledge comes from in-vitro studies with lymphocyte cell lines. Oestrogenes have a minor influence on lymphocyte proliferation in vitro, they, however, enhance antibody production and activate the promotor of the IFN-γ gene.[2] In contrast, progesterone downregulates the synthesis of cytokines in vitro.[3,4] In these experiments the effects of SH were only seen at supraphysiological concentrations.

We now investigated whether during normal menstrual cycle of healthy females there are variations of basal IL-6, sIL-6R and TNF plasma levels. In addition these cytokines were measured after ex-vivo stimulation of peripheral blood with LPS. For control the same parameters were determined in men and pregnant women. The results of our study suggest that progesterone may down regulate IL-6 during the menstrual cycle in vivo.

RESULTS

For the analysis of an effect of the menstrual cycle on cytokine production, five women were followed over 13 cycles, at least two cycles each. In Figure 1 the typical pattern of IL-6, IL-6R and hormones are shown from one woman followed over a period of four menstrual cycles. During the follicular phase the increase of β-estradiol was accompanied by an increase of IL-6 ($P = 0.07$, $r = 0.35$). After ovulation plasma progesterone levels increased about 10-fold and was accompanied by a 1.5–4.4-fold drop of the plasma IL-6 which again increases with the next cycle (Fig. 1). This pattern of inverse production for plasma IL-6 and progesterone was seen in 12 of 13 cycles (Wilcoxon test

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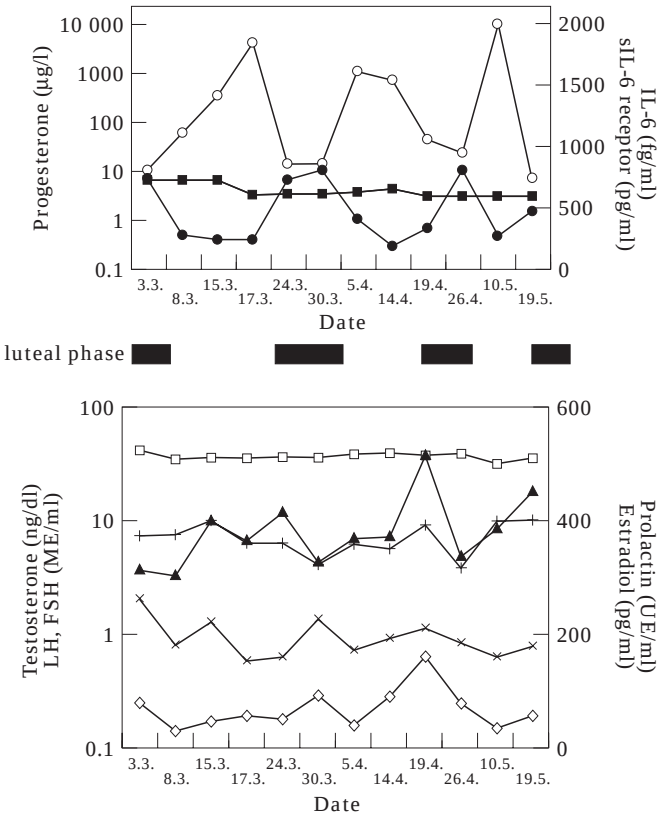

Figure 1. One woman was followed over four cycles and plasma IL-6, soluble IL-6 receptor and hormones were determined.

for matched pairs $P = 0.009$, $r = 0.526$) that were analysed in the five women (Fig. 2). Luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone, cortisole, growth hormone or prolactin failed to show any correlation to plasma IL-6 ($P > 0.05$). (Fig. 1).

One additional woman was excluded because of an irregular cycle without progesterone increase and without vaginal bleeding at the time. In this apparently healthy woman the amount of plasma IL-6 was about 10-fold higher than IL-6 of the other regularly cycling women.

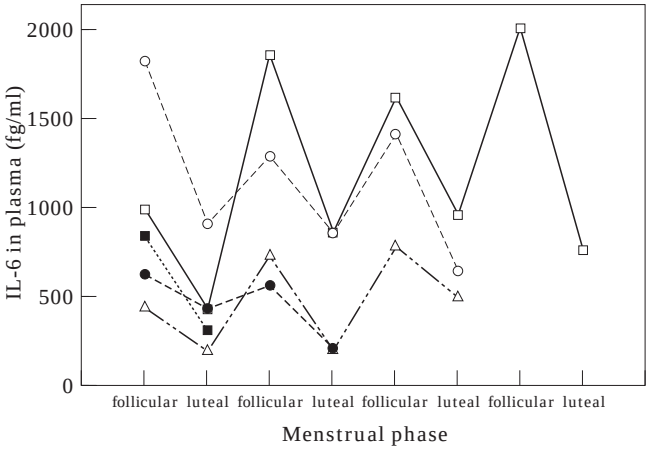

Figure 2. Five different woman were followed over some menstrual cycles and the maximum and minimum of the plasma IL-6 during follicular and luteal phase were plotted.

(○), woman 1; (●), woman 2; (□), woman 3; (■), woman 4; (△), woman 5.

Because progesterone has mineralocorticoid activity, it had to be discounted that the drop in the plasma IL-6 is caused by a difference in fluid balance. The haematocrit, total protein or albumin however, did not change significantly during the cycle (variability less than 10%, data not shown). Therefore, alterations of the plasma IL-6 during the hormonal cycle are not indirectly caused by changes in fluid balance.

During pregnancy, progesterone and β-estradiol are elevated up to 40-fold the range of pre-ovulatory women but without variations. In these five women basal IL-6 was in the range of the luteal phase of menstruating women without variations (Fig. 3). Even in men the plasma testosterone shows only minor weekly variations. In men there was no influence of testosterone on the basal IL-6 in plasma, even if one analyses 13 determinations in one single man.

When testing for biological activity in the 7TD1 proliferation assay no plasma IL-6 was detected (sensitivity of the assay 7.5 U/ml = 38 fg/ml). This indicates that immunoreactive IL-6 is either degraded or is not available for stimulation of cells due, for instance, to binding to the soluble IL-6 receptors (sIL-6R) which were found to be stable over the menstrual cycle (Fig. 1). Even during the normal menstrual cycle basal TNF or IL-10 (limit of sensitivity 15 pg/ml for TNF and 3 pg/ml for IL-10) were never found without further stimulation.

We next asked whether ex-vivo stimulation of whole blood would result in a similar pattern of IL-6 production during menstrual cycle. This study revealed no consistent pattern either for IL-6 (Fig. 4) or for TNF, i.e. the released cytokines after stimulation remained constant during the cycle. Furthermore, determination of blood monocyte subpopulations ($CD14^{++}$ and $CD14^{+}/CD16^{+}$ cells) by two colour flow cytometric assay and even the mean fluorescence intensity showed no fluctuations associated with the menstrual cycle (Fig. 4). Only in men with higher testosterone level there seems to be a pronounced IL-6 or TNF production after ex vivo stimulation ($P = 0.024$, $r = 0.48$).

DISCUSSION

In healthy women the menstrual cycle is regulated by periodical changes of SH and gonadotropines. Here we demonstrate for the first time that in vivo the plasma IL-6 varied during the cycle with an inverse pattern of plasma IL-6 and progesterone. The most important source of cytokine production in peripheral blood are the monocytes. In women isolated monocytes

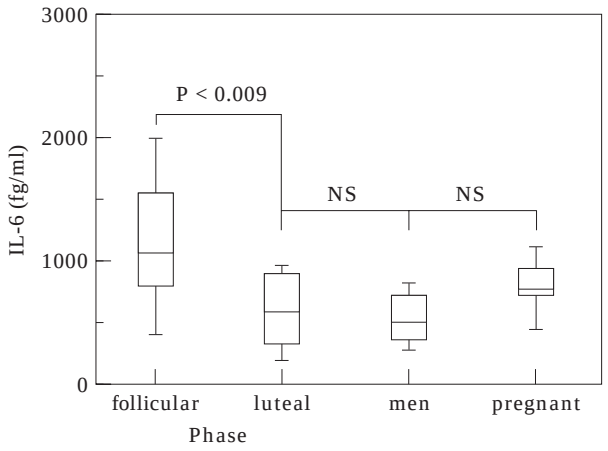

Figure 3. ($n = 9$) and women during luteal phase ($n = 5$) or pregnancy ($n = 5$) had similar plasma IL-6 levels lower than women in the follicular phase ($n = 5$).

produce higher amounts of IL-1 during the follicular than during the luteal phase without apparent stimulation.[5,6] During menopause, i.e. naturally occurring or induced after ovarectomy, an increased cytokine production by isolated PBMC in vitro could be reversed by the use of oestrogen or progesterone.[7,8] Even in men one can achieve a pronounced IL-1 synthesis of isolated PBMC with increasing amounts of oestrogen, but progesterone and testosterone had the opposite effect.[3,7] All these data were obtained with ex-vivo isolated and stimulated monocytes. Our results show that SH can have an impact on cytokine production in vivo, since we observed a pre-ovulatory increase of plasma IL-6 levels and a decrease with increasing progesterone during the menstrual cycle.

After specific stimulation of whole blood with LPS even with only 1 ng/ml the periodic changes could not be reproduced. The amount of stimulatory cytokines like IL-6 or TNF and the amount of the inhibitory cytokine IL-10 obtained with ex-vivo stimulation did not vary with SH and were always produced in comparable amounts. This is in agreement with the fact that the number of monocytes and granulocytes and the mean fluorescence of CD14 on monocytes or

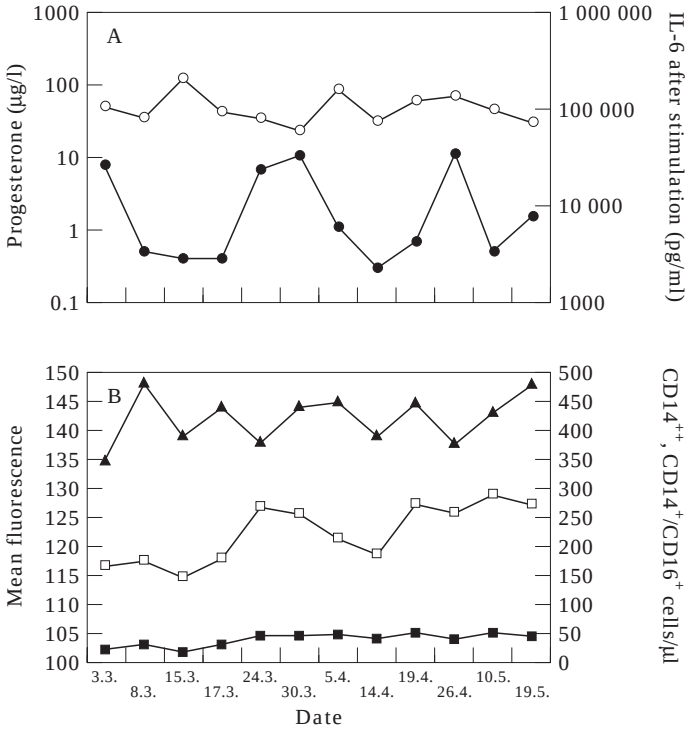

Figure 4.

A: Ex vivo production of IL-6 in whole blood after stimulation with 10 ng/ml LPS during the cycle of the same woman as in Figure 1; (●), progesterone; (○), plasma IL-6. [B: The number of CD14 strongly and weakly positive monocytes that covers CD16 (CD14/CD16$^+$ monocytes, 16) and the expression of the CD14–LPS-receptor on monocytes did not vary reproducibility during the cycle. (▲), LH; (+), FSH; (□), testosterone; (×), prolactin; (◇), estradiol.

(●), progesterone; (○), IL-6; (▲), mean flourescence of CD14^{++} cells CD14^{++}; (□), number of CD14^{++} cells/μl; (■), number of CD14^{++}/CD16$^+$ cells/μl.

granulocytes and therefore the amount of receptors for LPS[9] per cells did not change throughout the cycle (Fig. 4). The LPS activation of whole blood also stimulates cytokine synthesis in granulocytes but to a much lesser extent than in monocytes on a per cell basis. The high concentration of neutrophils in our whole blood assay might diminish effects of SH on the induced cytokine production of monocytes.

One source of the plasma IL-6 might be the uterus. However the proliferating mucosa cells in the uterus during follicular phase are unlikely to be the source of IL-6 because during pregnancy one would expect much higher amounts of IL-6 than during the follicular phase. Also plasma IL-6 is not a consequence of a local inflammatory reaction at time of menstrual bleeding, because plasma IL-6 was highest during the follicular phase and was inverse to progesterone which is low during menstrual bleeding. Therefore plasma IL-6 is unlikely to be derived from the uterus mucosa.

The granulosa cells in the ovary produce IL-6 under the control of gonadotropines.[10] IL-6 stimulates apoptosis of granulosa cells, suggesting that IL-6 might be involved in folliculogenesis. An elevated level of IL-6 might therefore have an impact on the development of the follicle in the ovary as seen in the woman we excluded because of irregular cycle. We did not find a constant correlation between the gonadotropine levels and IL-6 (Fig. 1).

The IL-6R itself has no transducing capacity, but signalling is mediated by a 130-kDa glycoprotein in the cell membrane. IL-6R is released from monocytes independently of the ligand by a shedding process that is regulated by protein kinase C.[11] This sIL-6R, a 55-kDa protein, can even bind IL-6 with the same affinity as the membrane receptor. sIL-6R may associate with gp130 and stimulate cells via this component.[12] In the plasma we detected the sIL-6R at concentrations ranging between 400–1000 pg/ml without any influence of the hormonal cycle. In contrast, the basal IL-6 with 26-kDa weight was found at a concentration of 200–1000 fg/ml. Assuming that one receptor can bind only one ligand, the sIL-6R is always present in molar excess and all IL-6 is bound. Therefore, variable IL-6 concentrations may be transmitted to all cells which possesses gp130 in their membrane. It is unclear why the plasma IL-6 was inactive in the mouse proliferation assay in vitro. Since it is possible that there is a species barrier this issue will have to be addressed in a homologous system.

IL-6 stimulates the antibody synthesis or the proliferation of T lymphocytes.[2] By this way IL-6 may promote an autoimmune disease and factors lowering IL-6 may have a beneficial effect. Epidemiologically it is known that autoimmune diseases with a female preponderence are exacerbated at times without balanced hormones. At the time of menarche and at beginning of the menopause the production of progesterone is insufficient and oestrogen has no counterpart. The amount of cytokine production of monocytes is increased at the early menopause and slowly decreases with time after menopause.[8] Because oestrogen upregulates the progesterone receptor, the effect of a therapy with progesterone can only be expected after an induction time with an oestrogen therapy. Therefore, we speculate that a therapy with combinations of oestrogen and progesterones may ameliorate autoimmune diseases.

In summary, our study demonstrates for the first time a periodic variation of constitutive IL-6 levels within the menstrual cycle. This suggest that fluctuations of SH during the menstrual cycle may influence immune responses in vivo.

MATERIALS AND METHODS

Donors and blood sampling

All donors were healthy with regard to history, physical examination and laboratory parameters, all were non-smokers without a known allergy, had a regular life style and did not take any medications. A total of ten woman and nine men were studied, five of the ten women were pregnant at weeks 19–32. The five women with regular menstrual cycles were followed over at least two cycles. The range of age was 22–34 in women, 24–35 years in men.

After fasting overnight and after a resting period of at least 15 min blood was drawn between 8 and 9 a.m. in the morning using a G21 needle (2 ml EDTA-blood, 5 ml plasma and 10 ml peripheral blood in a heparinized plastic syringe). In women with a regular 28-day menstrual cycle blood was drawn at day 3–5, 10, 14, 21, 28 after beginning of the cycle, in pregnant women and in men blood was taken at least two times with an interval of 1–2 weeks.

Control parameters and analysed hormones

The routine laboratory tests performed at the beginning of the study were total cell count, glucose, creatinine, urea nitrogen, aspartate aminotransferase, lactate dehydrogenase, bilirubin, albumin and total protein, C-reactive protein, cholesterol and triglyceride. During each follow-up total cell count, glucose, albumin, total protein and C-reactive protein were determined.

In addition, at every time point the concentrations of the following hormones were determined as recommended by the manufacturer: 17β-estradiol (RIA, Sorin, Düsseldorf, Germany), progesterone (RIA, Biermann, Bad Nauheim, Germany), testosterone (RIA, BykSantec, Dietzenbach, Germany), luteinizing and follicle-stimulating hormone (RIA, Seruno, Maia Clone, USA), prolactin, growth hormone and basal cortisol (fluorometric sandwich immuno assay, Wallac Oy, Turku, Finland and as published in Refs 13, 14).

In vitro stimulation

One blood sample was immediately centrifuged and stored at −80°C, four samples were incubated at 37°C for

4 h in an incubator with 5% CO_2. Stimulation of whole heparinized blood was done as described.[15] In brief, we stimulated with different concentrations of LPS (1, 10 and 1000 ng/ml LPS of *Salmonella minnesota*, Sigma, München, Germany), and one additional tube without LPS served as negative control. After centrifugations plasma was aliquoted and stored at $-80^{\circ}C$ until use.

FACS-analysis

The concentration of CD-14 positive cells in blood was calculated using flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) with the FITC labelled antibody My-4 and IgG_2B as a negative control (Coulter, Clone Krefeld, Germany) using identical concentrations. For double staining of monocytes[16] we used FITC-labelled CD14 and PE-labelled CD16 (Medac, Hamburg, Germany) in appropriate concentrations. EDTA-blood (100 µ) was incubated with labelled antibody or IgG_2B for 10 min at room temperature and processed according to the manufacturers instruction with the Coulter Immunoprep System to lyse erythrocytes, to fix the antibody and to stabilize the cells. This procedure was done during the first or second hour after blood was drawn. Ten thousand events were analysed without gating. The concentrations of CD-14 strong ($CD14^{++}$), CD14/CD16 positive monocytes and the specific mean fluorescence of the monocytes and granulocytes were determined.

Determination of cytokines

The cytokines TNF, IL-6, IL-10 and the soluble IL-6 receptor (sIL-6R) in plasma were analysed in commercially available sandwich ELISA (BioSource, Camarillo, California, USA). Since cytokines in plasma of healthy individuals are extremely low we used an ultrasensitive ELISA to determine the plasma IL-6 level (sensitivity 104 fg/ml) which detects IL-6 free and bound to sIL-6R. All determinations of a given individual had been done in a single kit and in duplicates as recommended by the manufacturer.

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