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Influence of menstrual cycle on NK activity

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Abstract

Natural killer (NK) cells are $CD3^-$ $CD56^+$ and/or $CD16^+$ cytotoxic lymphocytes that mediate first-line defense against various types of target cells without prior immunization. To assess the effect of the menstrual cycle and gender on NK activity we evaluated 30 healthy women (mean age 28.1 years, range 21–39) in follicular and luteal phases, 29 postmenopausal women (mean age 58.8 years, range 42–72) and 48 healthy men (mean age 31.6 years, range 21–40). In a flow cytometric test of NK activity, peripheral blood mononuclear effector cells were mixed with K562 targets cells labeled with DiO (3,3′-dioctadecyloxacarbocyanine perchlorate) at effector:target cell ratios of 40, 20, 10 and 5:1. Dead cells were stained with propidium iodide and results were expressed as lytic units per 10^7 cells. In addition, progesterone levels were determined in the luteal phase of the menstrual cycle of healthy women by a chemiluminescence assay. Our results showed that (1) NK cytotoxicity was higher in the follicular than in the luteal phase of the menstrual cycle ($P < 0.0001$); (2) postmenopausal women and men showed NK activity similar to women in the folicular phase but higher than women in the luteal phase of the menstrual cycle ($P < 0.05$); and (3) there was no correlation between NK activity and levels of progesterone. The data suggest that progesterone does not influence NK activity directly and that other factors may explain the reduction of NK activity in the luteal phase of the menstrual cycle.

Keywords: Natural killer; Menstrual cycle; Flow cytometry; Progesterone

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1. Introduction

Natural killer (NK) cells are $CD3^-$ $CD56^+$ and/or $CD16^+$ bone-marrow-derived lymphocytes that mediate cytotoxic activity against restricted target cells and produce significant amounts of cytokines and chemokines. NK cells are part of the first line innate defense system that acts against various types of target cells, such as tumor cells or virus-infected cells (Biron et al., 1999).

NK cell activity is positively regulated by interferon-γ, a key molecule for phenotypic differentiation and functional activation of these cells, and negatively modulated by glucocorticoids (Masera et al., 1999). In contrast, other hormones such as ACTH, β-endorphin and prolactin can enhance NK cytotoxicity (Gatti et al., 1993; Masera et al., 1999; Matera et al., 1999). On the other hand, there is controversy about the role of estrogen on NK activity. Seaman et al. (1978) reported that estrogen decreases NK activity in vivo; Sorachi et al. (1993) suggested that estrogen activates NK cells in vitro whereas progesterone does not influence NK activity. Finally, according to Sulke et al. (1985a) and to Uksila (1985) neither estrogen nor progesterone change NK activity in vitro.

Classically, NK activity is evaluated by measuring the ability of NK cells to kill special target cells such as the erythroleukemia cell line K562 labeled with ^{51}Cr sodium chromate (Brunner et al., 1968). Although this method has been accepted as the 'gold standard' for NK activity, it has been gradually replaced by non-radioactive methods based on fluorescent or immunoenzymatic technology (Korzeniewski and Callewaert, 1983; Krosen et al., 1992). In the present study we evaluated NK activity by flow cytometry in the peripheral blood of healthy subjects in order to investigate the effect of the menstrual cycle and gender on that activity.

2. Subjects and methods

2.1. Subjects

We evaluated NK cell activity of 107 healthy volunteers divided into three groups: Group I — 30 women with regular menstrual cycle (mean age 28.1 years, range 21–39), Group II — 29 postmenopausal women (mean age 58.8 years, range 42–72) enrolled according to the SENIEUR protocol (Ligthart et al., 1984) and Group III — 48 men (mean age 31.6 years, range 21–40). All patients were off any therapy at the time of the study. Postmenopausal women were in menopause at least for two years without using hormone replacement regimes, their estrogen levels were lower than

20 pg/ml, FSH levels were higher than 60 MIU/ml and endometrial biopsy showed an atrophic pattern. This investigation was approved by the Institutional Ethics Committee and informed consent was obtained from all subjects enrolled.

2.2. Effector cells

NK activity was measured in group I: (a) at the follicular phase (5th–8th day of the menstrual cycle) (Ia) and (b) at the luteal phase (22nd–26th day of the menstrual cycle) (Ib) and in one occasion in groups II and III. Ten milliliters of blood were collected in tubes containing 10 U/ml sodium heparin.

Mononuclear cells were separated from peripheral blood by Ficoll-hypaque density gradient centrifugation (Boyum, 1968), and adherent cells were removed (more than 95%) by incubation in a glass flask for 60 min at 37°C. Non-adherent cells were resuspended in RPMI 1640 medium with 10% fetal bovine serum (FBS — GIBCO) and adjusted to 1×10^9 cells/l.

2.3. Target cells

The human erythroleukemic cell line K562 was maintained in culture resuspended in RPMI 1640 medium with 10% FBS at 37°C in a humidified 5% CO_2 chamber. K562 cells were used for the NK assay in log phase diluted in a phosphate buffer saline (PBS) at 1×10^9 cells per liter. Cells were labeled with the stain 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, Molecular probes, Eugene, OR) for 20 min of incubation at 37°C and 5% CO_2.

2.4. Natural killer cell activity assay

NK activity against K562 target cells was assessed by a flow cytometry assay (Chang et al., 1993) using the DiO membrane dye to stain live K562 cells and propidium iodide (PI, Sigma, USA) nuclear dye to stain dead cells.

Unstained effector cells (NK) and stained target cells (K562) were added to each of six Falcon polystyrene 12×75 assay tubes at effector:target ratios of 40:1, 20:1, 10:1 and 5:1. Control tubes contained effector and target cells separately. Propidium iodide was added (13 mg/tube) and the tubes were incubated in a 5% CO_2 atmosphere at 37°C for 2 h. This incubation time showed the best correlation with the standard ^{51}Cr assay (Chang et al., 1993). Fluorescence of target cells (green) and dead cells (red) was evaluated in a flow cytometer (FACSort, Becton Dickinson Immunocytometry Systems, San Jose, USA) using two-parameter dot plots. Percentage of specific lysis was calculated by the formula:

$$\frac{\%\ \text{Dead target cells}}{100 - \%(\text{Target cell debris and fragments})} \times 100$$

NK activity was expressed by the number of lytic units 40% (LU 40%) per 10^7 cells for all groups.

2.5. Progesterone levels

Progesterone was quantified in the serum of patients from group Ib by a chemiluminescence method (De Boever et al., 1984, 1990). Intraassay error was 11% and sensitivity was 0.2 ng/ml.

3. Statistical analysis

Nonparametric matched paired Wilcoxon test was employed to evaluate differences in NK activity between follicular and luteal phases of the menstrual cycle in group I. Kruskal–Wallis test was employed to evaluate differences in NK activity among different groups (follicular phase, groups II and III; luteal phase, groups II and III). Spearman's correlation test was employed to analyze correlation between the levels of progesterone and NK activity in the luteal phase of the menstrual cycle. A $P < 0.05$ was considered significant.

4. Results

Women in the luteal phase of the menstrual cycle showed lower values of NK activity (median $\pm$ quartile amplitude $= 10.8 \pm 10.4$) compared to women in the follicular phase (median $\pm$ quartile amplitude $= 13.9 \pm 14.2$) of the menstrual cycle ($P < 0.0001$ — Fig. 1). Fig. 2 shows that there was no difference in NK activity between group Ia (women in the follicular phase of the menstrual cycle), group II (postmenopausal women — median $\pm$ quartile amplitude $= 13.6 \pm 14.2$) and group III (men — median $\pm$ quartile amplitude $= 13.2 \pm 13.8$). Women in the luteal phase of the menstrual cycle (group Ib) exhibited lower NK activity in comparison with postmenopausal women (group II) and men (group III), whereas group II did not differ from group III (Fig. 3).

There was no correlation between NK cytotoxicity and levels of progesterone (median $\pm$ quartile amplitude $= 6.5 \pm 4.9$) in the peripheral blood of women in the luteal phase of the menstrual cycle ($r = 0.18$; $P = 0.34$, Fig. 4). This is also true if we exclude the women with very low levels of progesterone (13 cases) indicative of the absence of ovulation (data not shown).

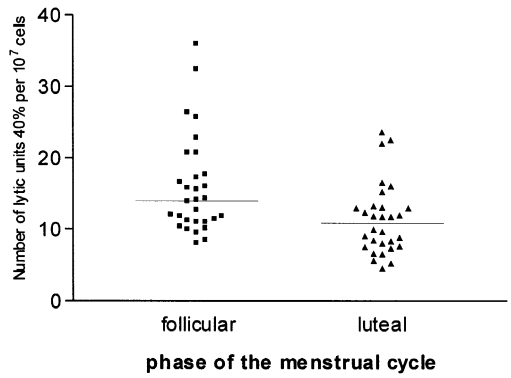

Fig. 1. NK activity according to the phase of the menstrual cycle. Horizontal bars represent medians. The difference between the two groups was statistically significant ($P < 0.0001$).

5. Discussion

In the present study we have demonstrated that healthy women in the luteal phase of the menstrual cycle have lower NK activity than women in the follicular phase of the menstrual cycle. NK activity in the follicular phase of the menstrual cycle was similar to that observed after menopause and in healthy men. Our data suggest that estrogen, a hormone that prevails in the follicular phase of the menstrual cycle, does not have a direct role in the modulation of NK function. This conclusion derives from the observa-

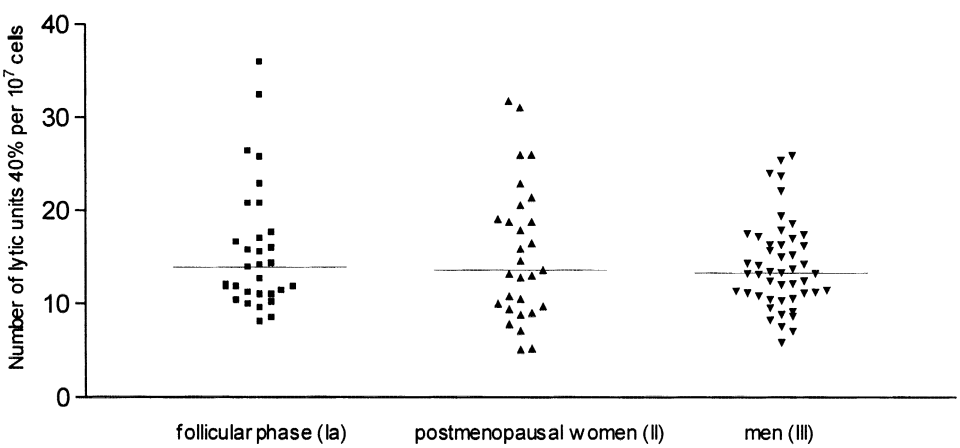

Fig. 2. NK activity in groups Ia (follicular phase of the menstrual cycle), II and III. Median is represented by horizontal bars. There was no statistical difference between the three groups.

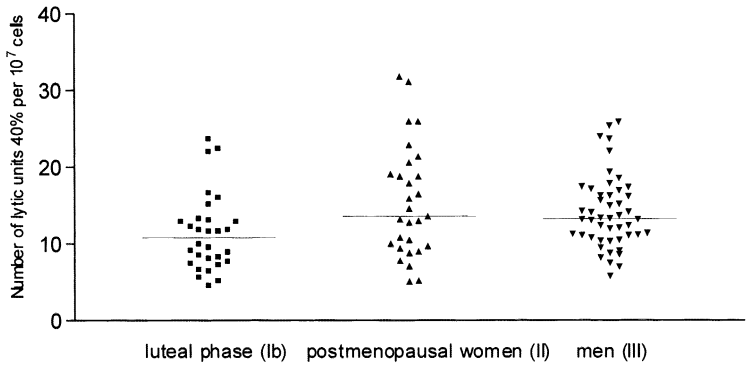

Fig. 3. NK activity in groups Ib (luteal phase of the menstrual cycle), II and III. Median is represented by horizontal bars. Statistical analysis performed by Kruskal-Wallis and Dunn test showed: Ib < II or III, II = III.

tions that (1) postmenopausal women who exhibit low levels of estrogen show NK function similar to women with normal levels of estrogen; and (2) health men who also exhibit low levels of estrogen show NK activity similar to women in the follicular phase of the menstrual cycle.

Some studies have demonstrated that elderly people have decreased NK activity (Facchini et al., 1987; Mariani et al., 1990), but others found normal NK activity (Ligthart et al., 1989) when they included only individuals who were in good health as described by the SENIEUR protocol (Ligthart et al., 1984). In our study the same protocol for selecting postmenopausal women was used, and our results show that women in good health reach advanced age with preserved NK function.

Sulke et al. (1985b) investigated NK activity in peripheral blood of 18 female volunteers at several stages during their menstrual cycle and showed

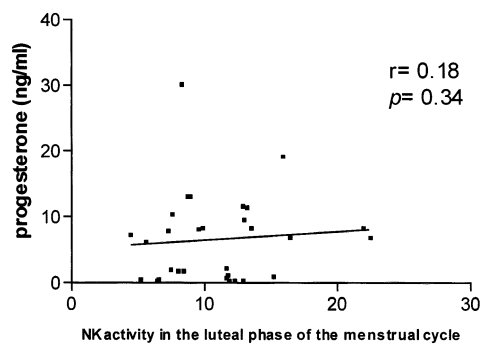

Fig. 4. Correlation between NK activity in the luteal phase of the menstrual cycle (group Ib) and levels of progesterone. Statistical analysis was performed by Spearman's test.

that estradiol in physiological concentrations did not affect directly the function of NK cells. On the other hand, Ferguson and McDonald (1985) showed that estrogen suppresses NK cytotoxicity in vitro, whereas Sorachi et al. (1993) suggested that estradiol was one of the activating factors of NK/LGL cells in vitro. The diversity of results could be due to small size of samples studied and the form of expression of results (specific cytotoxicity rather than lytic units).

Giglio et al. (1994) reported that the percentage of NK cytolysis was significantly lower (40%) in young women compared to young men (62%). However, in that study the peripheral blood was collected regardless of the phase of menstrual cycle. Addressing this point, Thyss et al. (1984) compared NK activity during follicular and luteal phases of the menstrual cycle and did not find significant differences between the two phases. However, only 13 patients were studied and the results of NK activity (6 cases increased, 6 reduced and 1 unchanged) were considered within the normal limits defined by the laboratory.

Since estrogens do not explain the variation of NK activity during the menstrual cycle, we investigated the role of progesterone in vivo. Our results failed to show a correlation between NK activity and the levels of progesterone in women in the luteal phase of the menstrual cycle. This observation suggests that progesterone does not play a major role in the modulation of NK activity. However, since progesterone secretion is pulsate, one value of this hormone may not be sufficient to reveal its effect on NK activity. In agreement with our data, Uksila (1985) and Sulke et al. (1985a) reported that in vitro incubation of human leukocytes with progesterone did not reduce NK activity. To the best of our knowledge, there are no other in vivo studies investigating the role of progesterone in NK activity.

In conclusion, we demonstrated that NK cytotoxicity changes during the menstrual cycle and it is reduced in the luteal phase compared to the follicular phase, to postmenopausal women and to healthy men. A decreased NK activity in the luteal phase of the menstrual cycle may be important to protect early pregnancy against immune aggression. Our data also suggest that progesterone does not influence NK activity directly, and that other factors, such as luteinizing hormone, inhibin-activin or cytokines, may explain the reduction of NK activity in the luteal phase of the menstrual cycle.

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