High NK cell activity in recurrent miscarriage: what are we really measuring?

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BACKGROUND: Several studies have shown that women with unexplained recurrent miscarriage (RM) have increased numbers and activity of peripheral blood NK cells and that elevated levels of these cells predict subsequent miscarriages in women with RM. Because catecholamines rapidly mobilize NK cells into the circulation, such increases may not reflect a steady state of overactive immunity but may result from a transient increase in the number of NK cells because of the stress associated with blood withdrawal. METHODS: Blood was drawn from 22 controls and 38 RM patients immediately after vein cannulation, and again 20 min later. The percentage of NK cells within lymphocytes, their concentration per microlitre of blood and their activity were assessed. RESULTS: All three indices of NK cells did not change in the controls across the two samples. However, women with RM had elevated levels in all three NK indices in the first blood sample, but these levels declined to values similar to those seen in the controls. This decline was mainly observed in primary aborters whose NK activity was highest in the first blood withdrawal. Accordingly, there was a high correlation between the magnitude of the decline and the initial NK cell indices in women with RM. The change in activity highly correlated with the change in the concentration of NK cells. CONCLUSION: The increased NK number and activity previously observed in RM patients may result from a transient stress response at the time of blood withdrawal. Patients with primary RM may be characterized by exaggerated acute stress responses in other circumstances.

Key words: NK cells/recurrent miscarriage/stress

Introduction

Several studies have shown that women with recurrent miscarriage have elevated NK cell numbers and activity both in the periphery (Aoki et al., 1995; Beer et al., 1996; Shakhar et al., 2003; Yamada et al., 2003) and in the endometrium (Clifford et al., 1999; Quenby et al., 1999) and that the levels of these indices predict subsequent pregnancy outcome (Aoki et al., 1995; Quenby et al., 1999; Emmer et al., 2000; Yamada et al., 2003). This elevation was mainly observed in primary aborters, for whom all pregnancies ended in miscarriage (Clifford et al., 1999), and less so in secondary aborters, who had a successful pregnancy followed by miscarriages (Shakhar et al., 2003). Studies in both humans and animals have attempted to link peripheral blood NK function to that of decidual NK cells, as decidual NK cells constitute 70% of uterine leukocytes in the luteal phase and the first trimester of pregnancy and are believed to play a role in pregnancy maintenance (Moffett-King, 2002). However, less than 10% of circulating NK cells resemble decidual NK cells in their surface markers (King et al., 1998; Searle et al., 1999; Hanna et al., 2003), cytokine secretion (Loke and King, 1995; Jokhi et al., 1997) and activity levels (King et al., 1989; Ferry et al., 1990; Ferry et al., 1991). Hence, the mechanism whereby circulating NK cells can predict miscarriage in RM is unclear.

The numbers of circulating NK cells are highly sensitive to acute stress (Landmann, 1992; Schedlowski et al., 1993; Benshop et al., 1996; Friedman, 1997; Benshop et al., 1998; Miller et al., 1999). Even mild psychological stress and moderate physical activity rapidly recruit large numbers of NK cells into the circulation, an effect which subsides shortly after the stress ceases (Dhabhar et al., 1995; Benshop et al., 1998). NK cells express high levels of β-adrenergic receptors (Landmann, 1992), and their adherence to endothelium and migration into the blood are affected by catecholamine levels (Benshop et al., 1996). Indeed, when physiologically relevant doses of adrenaline were infused to healthy human subjects in our laboratory (0.1 μg/kg/min, causing an approximately 15% increase in heart rate), NK cell counts increased two-fold within 5 min,
five-fold within 10 min and returned to baseline within 20 min of terminating the infusion (Shamgar Ben-Eliyahu, unpublished data).

Blood withdrawal or its context could be stressful. Thus, higher NK cell indices may result from a heightened stress response to blood sampling, rather than reflect a steady elevated state. To investigate these alternatives, we determined the numbers and activity of NK cells on blood samples drawn immediately after vein cannulation, and also 20 min later. If stress were responsible for the increased numbers and activity of NK cells, these indices would be expected to be lower in the second sample, after patients adjust to the situation.

Materials and methods

Sixty women participated in the study which was approved by the Human Investigation Review Board of the Sheba Medical Center, Tel Hashomer, Israel. All women gave written informed consent before their participation in the study.

NK indices were assessed in 38 women with at least three consecutive first-trimester miscarriages (mean ± SD age: 32 ± 5 years). Fourteen women were with primary RM (in whom all pregnancies had terminated as miscarriages) and 16 secondary RM (live birth followed by at least three consecutive miscarriages). Eight women were either with tertiary RM (miscarriages, live birth and subsequently at least three miscarriages) or their status was unknown. The average miscarriage number was 3.9 for primary aborters and 4.6 for secondary aborters. The control group consisted of 11 nulliparous and 11 parous women, none of whom had any known fertility problem. Table I summarizes mean age, number of miscarriages and children in the four groups. Scheffe post hoc tests revealed that primary and secondary aborters did not significantly differ in the number of miscarriages, and each group had an increased number of miscarriages compared to each of the control groups. Parous controls and secondary aborters had more children than nulliparous controls or primary aborters. With regard to age, only nulliparous controls were significantly different from secondary aborters.

Women with recurrent miscarriage were included after other presumptive aetiological factors were found to be normal: karyotype of both parents, glucose tolerance test, toxoplasmosis serology, hysterosalpingogram, thereby excluding anatomical abnormalities, intrauterine adhesions and cervical incompetence, thyroid function, serum phospholipid antibodies.

Experimental procedure

Blood was collected in 23 sessions. Approximately two women with RM and one control participated in each session. A cannula was inserted, and blood was collected immediately after cannulation, and again 20 min later. Venous blood was collected into heparinized (30 U/ml of blood) vacuum tubes between 9:00 and 12:00 a.m. Whole-blood NK cytotoxicity assays were performed on the day of blood collection. Whole blood was co-incubated with target cells within 2 h of drawing the last blood sample.

Radiolabelling of K562 target cells

Standard NK-sensitive K562 erythromyeloid tumour cells (Lozzo and Lozio, 1979) were used as targets in the NK cytotoxicity assay. Cells were grown in complete medium (CM) [RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μg/ml of gentamycin, 2 mM l-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (Biological Industries, Beit Haemek, Israel)] at 37°C in 5% CO₂. For radiolabelling, 4 × 10⁵ cells were incubated for 1.5 h with 200 μCi ⁵¹Cr, 200 μl of FCS and 150 μl of CM. Following incubation, cells were washed three times with CM (300 × g, for 10 min) and adjusted to the desirable concentrations.

NK cytotoxicity assays

Eight aliquots of 200 μl of whole blood (containing plasma, white and red blood cells) were placed in the wells of a microtitre plate. Target cells were then layered above the blood to achieve the eight different E : T ratios. A concentration of 2 × 10⁵ K562 cells per well was used for the lowest E : T ratio and consecutively diluted by two for higher E : T ratios. To determine spontaneous and maximal ⁵¹Cr release, whole blood was substituted with CM or Triton-100 (Sigma, Israel), respectively. Plates were centrifuged at 500 × g for 10 min and then incubated for 4 h in 5% CO₂ at 37°C. Following incubation, plates were centrifuged again, and 100 μl of the supernatant was recovered from each well for assessment of radioactivity in a γ counter.

The whole-blood procedure has previously been shown to reflect cytotoxicity carried out by NK cells and not by other cell types or serum factors (Ottenhof et al., 1981; Ree and Platts, 1983).

Flow cytometry

FACS (FACSAn, Becton Dickinson, San Jose, CA, USA) analysis was used to assess the number of NK cells in the blood. Fifty microlitres of whole blood was incubated for 45 min at 4°C with the following mouse anti-human antibodies: RPE-Cy5-conjugated anti-CD3, FITC-conjugated anti-CD6 and RPE-conjugated anti-CD56 (Dako, Glostrup, Denmark; Serotec, Oxford, UK). Erythrocytes were lysed using Lysing Solution (Becton Dickinson) and washed twice in phosphate-buffered saline containing 2% FCS and 2% Na₂HPO₄. A total of 20,000 events were acquired. NK cells were identified as CD3⁺CD16⁺, CD3⁺56⁺ or CD3⁺CD16⁺56⁺ lymphocytes. All analyses were conducted on the combined three subgroups of NK cells.

To assess the absolute number of NK cells per μl blood, a fixed number of polystyrene microbeads (20 μm, Duke Scientific, Palo Alto, CA, USA) were added to the blood samples before preparation for cytometric analysis. For more details see Shakhar et al. (Shakhar and Ben-Eliyahu, 1998).

Data analysis

Percent-specific lysis by NK cells

The following formula was used: (test cpm × correction – spontaneous cpm)/(maximal cpm – spontaneous cpm) × 100 where ‘correction’ is the percentage of the supernatant within the volume of the well (i.e. excluding the haematocrit volume). This correction is necessary because the chromium released by the labelled target cells is found only in the supernatant above the red blood cells (Ottenhof et al.,

Table I. Patients’ characteristics

<table>
<thead>
<tr>
<th></th>
<th>Parous controls</th>
<th>Nulliparous controls</th>
<th>Secondary recurrent miscarriage</th>
<th>Primary recurrent miscarriage</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Mean age ± SD</td>
<td>31.1 ± 3.5</td>
<td>28.6 ± 4.3</td>
<td>34.0 ± 5.1</td>
<td>29.1 ± 3.9</td>
</tr>
<tr>
<td>Number of miscarriages</td>
<td>0.3 ± 0.7</td>
<td>0</td>
<td>4.6 ± 1.4</td>
<td>3.9 ± 1.6</td>
</tr>
<tr>
<td>Number of children</td>
<td>1.9 ± 1.2</td>
<td>0</td>
<td>1.8 ± 0.9</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are mean ± SD.
Recurrent miscarriage, NK cells and stress

The relevant levels of maximal and spontaneous release were measured for each target cell concentration.

**Lytic units (LU)**

LU_{35} is the concentration of target cells (in thousands) at which there is 35% killing. The regression exponential fit method (Pollock et al., 1990) was used to calculate LU_{35} for each subject.

**Statistical analysis**

For statistical analysis, a repeated measures ANOVA was used. If significant group differences existed, Fisher’s PLSD analyses were conducted. The α level was set to 0.05 for all analyses.

**Results**

**Missing data**

The number and the percentage of NK cells in one primary aborter were lost because of a technical error. Additionally, the following three data points were removed from analysis, because they were more than 5 SD above the mean of their group: the concentration of NK cells per microlitre of blood of a primary aborter and of a parous control and the activity level of a primary aborter.

**Percentage of NK cells within lymphocytes, blood concentration of NK cells and NK activity in first and second blood withdrawals**

The first blood sample showed results which concurred with previous studies: women with RM exhibited a higher percentage of NK cells within lymphocytes ($F_{1,57} = 3.83, P = 0.05$), increased blood concentration of NK cells ($F_{1,55} = 9.29, P < 0.01$) and increased NK activity ($F_{1,57} = 5.81, P < 0.05$) compared to controls. However, when results from the second blood withdrawal were added to the analysis in a repeated measures design, significant interactions were observed (Figure 1): the magnitude of the decline between the first and the second blood withdrawal (Figure 2) was more than three-fold greater in RM patients than in controls for each of the NK indices [percentage of NK cells within lymphocytes ($F_{1,57} = 6.91, P < 0.05$), blood concentration of NK cells ($F_{1,55} = 7.79, P < 0.01$) and NK activity ($F_{1,57} = 11.66$)].

Figure 1. NK cell indices (mean ± SE) in the first and second blood withdrawal in women with recurrent miscarriage and controls. (A) Percentage of NK cells within lymphocytes. (B) Concentration of NK cells per microlitre of blood. (C) NK cell activity. All three measures were significantly higher during the first blood withdrawal but declined to levels similar to those in controls during the second blood withdrawal.

Figure 2. The difference in NK cell indices between the two blood withdrawals in women with recurrent miscarriage and controls. (A) Difference in percentage of NK cells within lymphocytes. (B) Difference in concentration of NK cells per microlitre of blood. (C) Difference in NK activity. Compared to control, the magnitude of the decline was over three-fold in women with recurrent miscarriage in each of the three indices. The dotted line represents the median of women with recurrent miscarriage and is higher than the values exhibited by most control women. Asterisks denote significant differences ($P < 0.05$).
NK indices in the control group did not change across the two blood withdrawals.

In contrast, no group or time differences were observed regarding the number of granulocytes or non-NK lymphocytes.

### NK activity per NK cell

In the NK cytotoxicity assay, activity is affected by the number of NK cells and by their individual activity levels. When the activity per individual NK cell was analysed, no differences were observed between patients and controls in either the first or the second blood withdrawal, suggesting that NK activity per NK cell does not account for the differences in NK activity between the two groups.

### Correlations between NK indices in the first and second blood withdrawals

Among RM patients, the magnitude of the decline positively correlated with the initial values: $r = 0.87$ for the percentage of NK cells within lymphocytes ($F_{1,35} = 65.41, P < 0.0001$), $r = 0.79$ for blood concentration of NK cells ($F_{1,33} = 56.12, P < 0.0001$) and $r = 0.66$ for NK activity ($F_{1,35} = 26.82, P < 0.0001$). These correlations indicate that women with higher initial values showed a greater decline following the rest period.

Additionally, the correlation between the magnitude of the decline in activity and the magnitude in the decline of blood concentration of NK cells in women with RM was 0.81 ($F_{1,33}=60.91, p < 0.0001$), suggesting that the decrease in activity by the second blood withdrawal at least partly resulted from a decline in numbers of NK cells.

### NK indices in primary and secondary aborters

When the control group and the RM group were separated into those having children and those who do not have children (i.e. parous controls, nulliparous controls, secondary aborters and primary aborters), significant interactions were observed between the group and the timing of the blood withdrawal with regard to the concentration of NK cells ($F_{3,45} = 2.85, P < 0.05$) and their activity ($F_{3,46} = 3.53, P < 0.05$) (Figure 3), but not with regard to their percentage. When the difference between the second and first blood withdrawal was analysed in post hoc analyses using Fisher’s PLSD, primary aborters (but not secondary aborters) had higher indices compared to each of the control groups in the concentration of NK cells as well as in their activity ($P < 0.05$). These suggest that the difference between controls and RM can be mainly attributed to primary aborters rather than to secondary aborters.

### Discussion

This study indicates that immediately after vein cannulation for blood withdrawal, women with RM show an increased proportion of NK cells within lymphocytes, elevated blood NK cell concentrations and augmented NK activity per millilitre of blood compared to control women who have no known fertility problems. However, these differences disappear 20 min later, when blood is drawn again from the same cannula. These declines are mainly seen in primary aborters, whose NK levels were the highest in the first blood withdrawal, and can be largely explained by changes in NK cell numbers. The levels of NK indices did not change across the two blood withdrawals. Overall, these results suggest that the elevated NK indices previously observed in RM patients are due to a transient increase in NK cell numbers, rather than a chronic state.

The question therefore arises as to what is really measured when high NK activity is found in women with RM. It is our hypothesis that an immune response to a transient sympathetic discharge of catecholamines is being measured. Catecholamines are known to immediately increase the numbers of circulating NK cells, and the kinetics of their rise and fall in response to an acute stressor corresponds well with the time course of our findings (Benschop et al., 1996). Although RM patients may exhibit a greater sympathetic response than controls because they perceive the blood test as critical for their diagnosis, this is probably not the main cause for the observed difference between the two groups. NK activity not only distinguishes RM patients from controls but has also been reported to predict future miscarriages within the population of unexplained RM (Emmer et al., 2000; Yamada et al., 2003; Aoki et al.,

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**Figure 3.** NK cell indices (mean ± SE) in the first and second blood withdrawals in nulliparous controls, parous controls, primary aborters and secondary aborters. (A) Percentage of NK cells within lymphocytes. (B) Concentration of NK cells per microlitre of blood. (C) NK activity. A significant interaction was observed between group and the timing of the blood withdrawal. Specifically, primary aborters, but not secondary aborters, had greater declines between the two blood withdrawals than each of the control groups.
1995; Quenby et al., 1999). Thus, the increase in NK indices is probably more than a specific response to the blood test and may be representative of the response of these women to other stressors they encounter in everyday life. It is possible that a greater sympathetic response to stressors elevates the risks of miscarriage as well as leads to an enhanced increase in NK cell indices after an exposure to a stressor. These women could have been more responsive to stressors even before they had miscarriages, or they may have acquired it parallel to their experience of recurrent miscarriage. The notion that psychological stress is associated with RM has been previously shown in several studies (Liddell et al., 1991; Friedman, 1997; Aoki et al., 1998), and psychological support was shown to lower miscarriage rates in this population by up to three-fold (Tupper and Weil, 1962; Stray-Pedersen and Stray-Pedersen, 1984; Liddell et al., 1991; Clifford et al., 1997). The mechanism through which stress could affect miscarriage is unclear, but neuroendocrine modulation of uterine immunity function by stress responses may be involved. Future studies should assess whether RM patients are more responsive to stressful stimuli, whether they exhibit elevated sympathetic responses and whether these responses predict pregnancy outcome.

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