Peripheral natural killer cytotoxicity and CD56<sup>pos</sup>CD16<sup>pos</sup> cells increase during early pregnancy in women with a history of recurrent spontaneous abortion

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For diagnostic purposes we assessed peripheral natural killer (NK) cell cytotoxicity and NK and T cell numbers to assess their putative predictive value in recurrent spontaneous abortion (RSA). A total of 43 women with subsequent pregnancy, 37 healthy controls and 39 women successfully partaking in an in-vitro fertilization (IVF) procedure, were included in the study. We show that before pregnancy, levels of NK cytotoxicity and numbers of both single CD56<sup>pos</sup> and double CD56<sup>pos</sup>CD16<sup>pos</sup> cells were similar between RSA women and controls. But notably, within the RSA group, NK cell numbers of <12% were strongly associated with a subsequent pregnancy carried to term. Supplementation of folic acid led to an increase of single CD56<sup>pos</sup> cells, but cytotoxic function appeared unaffected. The expression pattern of killer inhibitory receptors on CD56<sup>pos</sup> cells was not different between patients and controls. A longitudinal study revealed that, compared with controls, in RSA women higher numbers of double CD56<sup>pos</sup>CD16<sup>pos</sup> cells were present during early pregnancy, paralleled by an increase in cytotoxic NK cell reactivity. The single CD56<sup>pos</sup> population decreased in number. In conclusion, the analysis of peripheral NK cell characteristics appears a suitable diagnostic tool in RSA. Immuno-modulation aimed at NK cell function appears a promising therapeutic measure.

Key words: abortion/CD56/folic acid/KIR/natural killer cells

Introduction

About 0.5–1% of couples who are trying to conceive will be suffering from subsequent spontaneous miscarriages (Regan, 1992). Some 80% of those losses cannot be accounted for by chromosomal defects, hormonal disorders or uterine abnormalities. Evidence is accumulating that these unexplained miscarriages might have an immunological background. The presence of fetal cells in the maternal circulation (Van Wijk et al., 1996) shows that the fetal–maternal interface is not an absolute barrier. Since the fetus is semi-allogeneic to the mother and it has conclusively been shown that both fetal cells and placental tissues do express the paternally derived and thus foreign HLA antigens (Houlihan et al., 1995; King et al., 1996; Hutter et al., 1996), it can safely be assumed that every successful pregnancy must be accompanied by redirection or suppression of natural killer (NK) and T cell reactivity. Fine tuning appears to be an essential feature of the fetal–maternal interaction. The consequences of unbalance appear to be particularly exemplified by the contrasting findings in endometriosis as compared with RSA patients (Somigliana et al., 1999). Apparently, excessive immunosuppression (the Th2 environment observed in endometriosis) leads to uninhibited endometrial growth, with unfavourable outcome. In turn, a greater activation or reduced immunosuppression (favouring a Th1 environment, including cytotoxicity) leads to insufficient growth and maturation. Indeed, elevated expression of T cell activation markers has been associated with early pregnancy loss (Vassiliadou et al., 1999). HLA antigens might well play a pivotal role in this process by not only supporting fitness (by exerting influence on survival and reproductive success), but also development (Fernandez et al., 1999).

The notion that the immune system requires modulation in order to ensure successful pregnancy has in the case of RSA already led to preventative measures, e.g. i.v. injection of immunoglobulin (IgG) (Rigal et al., 1994; Ruiz et al., 1996) and paternal leukocyte immunization (Daya and Gunby, 1994; Check et al., 1997).

Of particular interest is the role of NK cells. During early pregnancy the so-called uterine NK cells form the major population of immune competent cells in the maternal–fetal interface. These cells carry inhibitory receptors for HLA antigens, most notably CD94/NKG2a, CD158a and CD158b (King et al., 1997a; Ponte et al., 1999). At the placental interface HLA-G is the major HLA antigen with prolonged and significant levels of protein expression, but (transient) expression of HLA-C has also been observed (Hutter et al., 1996; King et al., 1996). In-vitro studies with both uterine and peripheral NK cells have shown that the cytotoxic reactivity against HLA-G expressing targets of distinct origin was inhibited (Rouas Freiss et al., 1997; Rajagopalan and Long, 1999). This suggests that the interaction of HLA antigens and NK cells at the placental interface could be critical in determining the outcome of pregnancy. So far, few groups have studied peripheral NK cell characteristics in RSA women. Beer, Kwak and co-workers have shown that before and during pregnancy NK cell numbers in RSA women are elevated, whereby high numbers appeared predictive of subsequent miscarriage (Kwak et al., 1995; Beer et al., 1996). Also, evidence has been obtained that NK cell cytotoxic activity is increased in RSA women (Aoki et al., 1995; Higuchi et al., 1995; Ruiz et al., 1996), particularly in those with subsequent...
pregnancy failure. Intravenous IgG treatment and paternal leukocyte immunization have already successfully been used to down-regulate NK cell activity (Higuchi et al., 1995; Kwak et al., 1996; Ruiz et al., 1996).

We here report the results of a longitudinal study comprising pre- and post-conceptional data on both NK cell cytotoxicity and NK cell phenotypes in RSA women with either a successful or failed pregnancy. Thus, we were able to answer whether pre-conceptional NK cell parameters differ between RSA women and controls and whether these are indicative of subsequent miscarriage. We assessed the changes in NK parameters within groups from pre- to post-conceptional stages and how these values differed between RSA women and controls at several points during the course of pregnancy. In the course of this study we also assessed the effect of folic acid intake on NK cell characteristics.

**Materials and methods**

**Study group and controls**

Over a 2 year period, 142 women with a history of RSA and in part previously described (Nelen et al., 1997, 1998) were entered into the study together with intake and follow-up controls. In the final retrospective analysis, including pre- and post-conceptional parameters, only RSA and follow-up control women with consecutive pregnancy were included. Inclusion criteria for RSA women were a history of at least two consecutive idiopathic miscarriages with the same partner and a wish for pregnancy. A miscarriage was defined as a spontaneous pregnancy loss before 16 weeks menstrual age. Women were excluded in case of chromosomal rearrangement in either partner, Lupus coagulant, antiphospholipid antibodies, or medication used to treat disorders other than vitamin B6 and B12 deficiency, diabetes mellitus, polycystic ovarian syndrome or thyroid dysfunction. Upon intake a first blood sample was drawn. Subsequently, women were given folic acid supplementation (0.5 mg daily) and after a 2 month period a second blood sample was drawn. As intake controls 37 non-pregnant healthy control women with at least one successful pregnancy and no history of RSA were entered into the study. As follow-up controls 39 women successfully partaking in an IVF procedure were entered. These women also received folic acid supplementation and were followed from the pre-conceptional period throughout successful pregnancy. Follow-up control women who miscarried were excluded. After pregnancy was established by urinary HCG (ICON test; Hybritech, Liege, Belgium) in both RSA women and follow-up controls, blood samples were drawn at 8 and 12 weeks of menstrual age, unless miscarriage occurred previously. From the 43 RSA women who became pregnant, 26 carried to term and 17 suffered from a recurrent miscarriage. The RSA women experienced a median of three consecutive miscarriages with a range between 2 and 8, 18 out of 43 women had a history of two consecutive miscarriages. The mean age of RSA women (31.9; 95% CI: 30.3; 33.6) did not differ from intake control women (mean 32.1, 95% CI: 30.6; 33.6) or follow-up control women (mean 33.0, 95% CI: 32.0; 34.1) (t-test, \(P > 0.05\)). The Institutional Review Board of the University Hospital Nijmegen approved the study and all women gave written informed consent, before participation.

**NK cell cytotoxic reactivity**

From all blood samples, peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation (Lymphoprep®, Nycomed, Oslo, Norway) and stored in liquid nitrogen until use. NK cell reactivity was assessed using a \(^{51}\)Cr release bioassay. In short: effector cells were thawed and incubated overnight in medium containing RPMI 1640 Dutch modification (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (PAA, Linz, Austria), 0.02 mmol/l sodium pyruvate, 100 U/ml penicillin and 100 \(\mu\)mol/l streptomycin (both Life Technologies); \(1 \times 10^5\) K562 target cells were labelled with 3.7 MBq \(^{51}\)Cr (Amersham, UK) for 2 h. Then, \(1 \times 10^3\) labelled target cells were added to 80 \(\times 10^3\), 40 \(\times 10^3\), 20 \(\times 10^3\) or 10 \(\times 10^3\) of effector cells resulting in four different effector/target (E/T) cell ratios (80:1, 40:1, 20:1, 10:1). Incubation was for 4 h in a humidified incubator at 37°C and 5% CO\(_2\). After incubation, 100 \(\mu\)l of supernatant was harvested and radioactivity was measured using a \(\gamma\)-counter (Wallac 1470 \(\gamma\) counter, Turku, Finland). The percentage specific NK cell cytotoxic reactivity was determined as follows:

\[
\% \text{ lysis} = \frac{\text{sample value} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}
\]

In order to express cytotoxic reactivity independent of an arbitrarily selected E/T ratio, and to allow for reliable comparison between samples of distinct individuals tested at different times, the specific lysis of the four E/T ratios per sample were conversed to lytic units (LU)(Bryant et al., 1992). The LU was defined as the number of times that an amount of effector cells, that gives rise to a specific lysis of 20%, was contained in 10\(^3\) effector cells (LU = \(10^3/\text{number of cells yielding 20\% lysis}\)). We therefore calculated the median of the logarithmic transformed specific lysis of the four E/T ratios of each tested sample the number of cells yielding 20% specific lysis, thus creating a robust method to compare different samples without disproportionate effects of occasional erratic data.

**Flow cytometric labelling and analysis**

Cells were phenotypically analysed by a two-step double labelling procedure. Briefly, cells were washed three times with fluorescence activated cell sorting (FACS) buffer [phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA)] and labelled first with (un)conjugated specific antibody, if necessary followed by conjugate binding (GAM–FITC; Dako, Glostrup, Denmark). Thereafter the cells were labelled with a secondary conjugated specific antibody. All incubations were for 30 min and thereafter the cells were washed twice. The samples were run on an XL-Epic’s (Coulter Electronics) and 5000 or 10,000 events were collected based on live lymphocyte cell gating as indicated by propidium iodide (5 \(\mu\)g/ml) staining. Isotype matched antibodies were used to define marker settings, isotype matched controls were usually below background staining. Data were analysed by Coulter XL-2 and/or WINMDI software. For analyses of NK cell (subset) numbers conjugated \(\alpha\)CD16-FITC (clone DJ130c), \(\alpha\)CD56-RPE (clone MOC-1), \(\alpha\)CD3-FITC or \(\alpha\)CD3-RPE (clone UCHT1) antibodies were used (all from Dako, Glostrup, Denmark). NK cell inhibiting/activating receptor expression was characterized by conjugated \(\alpha\)CD158a-PE (clone Eb6), \(\alpha\)CD158b-PE (clone GL183) and unconjugated \(\alpha\)NKG2a (clone Z199) antibodies (all from Immunotech, Marseille, France). Isotype matched control antibodies were directed against *Aspergillus niger* (Dako, Glostrup, Denmark).

**Statistical methods**

The \(t\)-test for independent samples was used to test for statistical significance of the differences in the mean values of the age and NK cell parameters between, first, the RSA group and the intake controls and, second, the RSA group and follow-up controls. The mean values with 95% confidence intervals (CI) are presented.

The \(t\)-test for paired samples was used to test for statistical
Effects of pre-conceptional folic acid supplementation in the RSA women

Since RSA women with a wish to conceive received folic acid treatment upon entering the clinic, we analysed whether the pre-conceptional intake of folic acid was associated with a subsequent change in NK cell parameters. A comparison of peripheral NK cell levels and activity before and during folic acid treatment within the RSA group (Table I) revealed no statistically significant change in cytotoxic activity. LU levels were 411.0 (95% CI: 378.1; 44.40) before and 393.1 (95% CI: 362.2; 423.9) during treatment (P = 0.185). Also, neither the total number of NK cells nor the number of T cells changed substantially. However, when looking at NK cell subsets, we did observe a significant rise in single CD56pos NK cells, from 1.4% (95% CI: 0.7; 2.2) up to 2.4 (95% CI: 1.5; 3.4) associated with a significant change in the ratio single CD56pos/double CD56pos/CD16pos cells from 0.25 (95% CI: 0.06; 0.44) up to 0.55 (95% CI: 0.28; 0.82) (P = 0.008).

Post-conceptional peripheral NK cell reactivity and cell numbers

We were also interested in finding out whether during the first trimester of pregnancy in RSA women (including both women
Figure 1. The estimated mean natural killer (NK) cell cytotoxic reactivity (LU) and NK cell parameters in recurrent spontaneous abortion (RSA) women (□) and follow-up controls (●) at each assessment point, using the method of last observation carried forward (LOCF). The vertical lines indicate the 95% confidence interval.

who carried to term as well as those that aborted) NK and T cell parameters underwent distinct changes in comparison with controls.

Figure 1 shows the course of NK cell cytotoxicity levels and cell numbers starting from pre-conceptional values up to 12 weeks of menstrual age, in both RSA women and controls. From Figure 1 it is clear that differences between the groups are most prominent at week 8 of menstrual age. To be better able to evaluate the differences between the groups, the estimated values as derived from the repeated measurements model are depicted in Table II. The differences were calculated for the overall period (column 2), and for each of the assessment points separately (columns 3 and 4). Adjusted for the pre-conceptional values, it appeared NK cell cytotoxicity (expressed as lytic units) at week 8 was significantly increased in the RSA group as compared with the controls [a difference of 55.2 (95% CI: 10.6; 99.8)]. But this difference tapered off and was almost negligible at week 12 [6.9 (95% CI: –37.1; 50.9)]. Similarly, NK cell levels in RSA women were higher during pregnancy [an overall difference of 4.0% (95% CI: 0.4; 7.6)], mainly due to a higher level of double CD56posCD16pos cells. This effect was again most prominent at week 8 of menstrual age [5.4% (95% CI: –0.2; 11.0)] but in this case was maintained at week 12. Levels of single CD56pos cells were lower in RSA women as compared with controls, both at weeks 8 and 12. In combination with the higher levels of double CD56posCD16pos cells this resulted in a notable reduction in the ratio single CD56pos/double CD56posCD16pos NK cells [–0.65 (95% CI: –1.0; –0.3)]. Notably, the concomitant rise of NK cell cytotoxicity, increased numbers of CD16pos NK cells and decreased levels of single CD56pos cells fits the known characteristics of NK cell activation.

CD3pos cell numbers (including the CD3posCD56pos cells) remained unchanged during pregnancy (data not shown).

Risk factors for miscarriage within the RSA group

Although all RSA women had a history of miscarriages, two-thirds of these women successfully carried their next pregnancy to term. Consequently, we looked for parameters that could predict the pregnancy outcome in the group of RSA women. The effect of the following parameters, measured before pregnancy, on the probability of a miscarriage was studied using univariate logistic regression analysis in the group of RSA women with two or more spontaneous abortions: age, LU, % NK cells, % double CD56posCD16pos cells, % single CD56pos cells, ratio single CD56pos/double CD56posCD16pos.
cells, % CD3<sup>pos</sup> cells, % double CD3<sup>pos</sup> CD56<sup>pos</sup> cells. Of these parameters only age proved to be a substantial risk factor with a 37% increased risk of miscarriage with each additional year of age (OR = 1.37; 95% CI: 1.07; 1.92; P = 0.03).

In addition, using the data from the regression analysis, we dichotomized the data for the above mentioned parameters and analysed both women with two or more abortions and those with three or more abortions. Table III shows the results for the pre-conceptional values (women were on folic acid).

Notably, using a threshold value of 12% (Coulam et al., 1995), in non-pregnant RSA women on folic acid treatment a low NK cell percentage was significantly associated with a successful subsequent pregnancy (P = 0.02; Fisher's exact test). In fact, all of the eight RSA women with pre-conceptional NK cell numbers <12% had subsequent successful pregnancies. In the group of women with three or more miscarriages the effect was similar, but due to low numbers not significant.

None of the other parameters tested revealed a significant these parameters only age proved to be a substantial risk factor with a 37% increased risk of miscarriage with each additional year of age (OR = 1.37; 95% CI: 1.07; 1.92; P = 0.03).

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**KIR/KAR and NKG2a expression on CD56<sup>pos</sup> cells**

Both uterine and peripheral NK cells can be activated or inhibited via specific surface molecules. The killing inhibitory/activating receptors CD158a, CD158b and NKG2a are examples of such surface molecules and are thought to interact with their ligands, HLA-C and HLA-E (presenting processed HLA-G), respectively. These receptors are known to be expressed at the fetal–maternal interface. In order to ascertain whether there are differences in NK cell inhibitory receptor repertoire between RSA women and controls, we determined the expression of CD158a, CD158b and NKG2a receptors on peripheral NK cells by flow cytometry (as shown in Figure
Expression was similar for all groups studied. CD158a, CD158b and NKG2a were expressed on approximately 20, 30 and 45% of CD56pos cells respectively.

**Discussion**

Recently, attention has focused on the role of uterine NK cells as mediators in successful implantation and placental maturation (Loke and King, 1997; King et al., 1997b). It is thought that these cells exert their function through cytokine production rather than cytotoxicity. In fact, the latter function should be inhibited to prevent pregnancy failure. Notwithstanding the fact that spontaneous miscarriage is a typically localized process, it is of great interest to evaluate systemic changes that precede this process, in particular for future diagnostic purposes. With this objective we performed a longitudinal study on peripheral NK cell parameters and were able to define that within the group of RSA women pre-conceptional CD56pos levels of less than 12% were associated with a successful subsequent pregnancy. In fact this confirms previous data (Kwak et al., 1995) which showed that high NK cell levels are associated with RSA and subsequent miscarriage. This held true for women with either two or more as well as for those with three or more spontaneous miscarriages.

Notably, before pregnancy we could not detect a significant difference in either NK cell cytotoxicity or numbers of double CD56posCD16pos cells between RSA women (grouped irrespective of the outcome of their next pregnancy) and controls, but we did observe a marked difference between these groups in early pregnancy. Whereas both cytotoxicity and NK cell numbers increased in the RSA group with advancing pregnancy, peaking at week 8 menstrual age, the opposite effect was that in women with a history of recurrent spontaneous abortion (RSA), RSA women have markedly increased NK cell cytotoxicity, RSA women have considerably increased NK cell numbers in RSA women, and this effect is not only important in the RSA group but also in women with recurrent spontaneous abortion. The fact that in our study pre-conceptional cytotoxic reactivity or NK cell numbers in RSA women could not be linked to a higher risk of miscarriage as was previously shown (Aoki et al., 1995; Coulam et al., 1995) cannot so easily be explained. In any case directly after conception these parameters reached significantly higher levels in RSA women compared to controls. Together with the fact that within our group of RSA women pre-conceptional NK cell levels were found to be predictive of subsequent outcome, the observations suggest that these cells are either directly or indirectly involved in the process of spontaneous abortion.

As regards the intake of folic acid, we found a positive effect of daily supplementation on single CD56 pos cells in RSA women. This was associated with a slight, but not significant drop in pre-conceptional cytotoxic reactivity. Folic acid is capable of lowering plasma homocysteine levels (Nelen et al., 1998), and a high plasma level of this protein is a known risk factor for reproductive disorders such as neural tube defects, placental pathology, pre-eclampsia and RSA (Obwegeser et al., 1999). It is not yet clear whether the effects of folic acid on reproductive performance are limited to the homocysteine pathway or are in fact more extensive. Based on our data it would be of interest further to elucidate the role of folic acid on NK cell function.

In conclusion, we have found evidence for the hypothesis that in women with a history of recurrent spontaneous abortion, low pre-conceptional peripheral NK cell levels are indicative for a subsequent successful pregnancy. During pregnancy, RSA women have markedly increased NK cell cytotoxicity, associated with a rise in double CD56posCD16pos cells and a concurrent drop in single CD56pos cells. Thus in RSA, diagnostic and therapeutic measures aimed at characterizing and modulating NK cell activity appear promising in the future.

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**Table III. Retrospective analysis of putative risk factors for subsequent miscarriage within recurrent spontaneous abortion (RSA) women**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Threshold value</th>
<th>Successful pregnancy</th>
<th>Miscarriage</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lytic units</td>
<td>&lt;322</td>
<td>4 (3)</td>
<td>3 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>% CD56posCD3neg</td>
<td>&lt;12.0</td>
<td>8 (5)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>% CD56posCD16posCD3neg</td>
<td>&lt;20.7</td>
<td>12 (8)</td>
<td>7 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>% CD56posCD16posCD3neg</td>
<td>&lt;4.0</td>
<td>14 (8)</td>
<td>5 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>CD16pos</td>
<td>&lt;0.08</td>
<td>5 (3)</td>
<td>2 (1)</td>
<td>NS</td>
</tr>
<tr>
<td>CD16pos</td>
<td>≥0.08</td>
<td>10 (6)</td>
<td>5 (5)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant.

*aNumber of RSA women with successful pregnancy or subsequent miscarriage after dichotomizing using the specific threshold values. Values in parentheses are the numbers of RSA women with three or more subsequent miscarriages.
Peripheral NK cell reactivity in RSA


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