Detailed analysis of peripheral blood natural killer (NK) cells in women with recurrent miscarriage

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BACKGROUND: Increased peripheral blood natural killer (NK) cell activity has been associated with unexplained reproductive failure including recurrent (three or more) miscarriages (RM). Studies have reported abnormalities in both numbers (absolute and proportion) and activation. This study assessed numerous NK cell parameters to determine which (if any) are altered in women with RM compared with controls, which parameter best differentiated women with RM from controls, and what NK levels should be considered high.

METHODS: Luteal-phase blood samples from women with RM (n = 104) and controls (n = 33) were analysed by four-colour flow cytometry. NK cells were analysed as a percentage of lymphocytes, total NK concentration, CD56^Dim subtype concentration and percentage, activated CD69^+CD56^Dim subtype concentration and percentage and CD56^Bright:CD56^Dim subtype ratio. Women with RM were analysed in two subgroups: those positive in ≥1 RM screening tests (karyotype, uterine, antiphospholipid syndrome, thrombophilia) (n = 48) and those who had negative screening tests (n = 56).

RESULTS: Women with RM had significantly higher NK percentage (P < 0.001), and significantly lower CD56^Bright:CD56^Dim ratio (P < 0.05) than controls. NK percentage was the only significantly higher variable in the RM screening test negative subgroup (P < 0.01). A ROC analysis (AUC = 0.71) found that an NK percentage >18% was highly specific for women with RM (97.0%), and defined 12.5% of women with RM as having high NK percentage, compared with 2.9% of controls.

CONCLUSION: Women with RM have altered peripheral blood NK parameters. NK cells as a percentage of lymphocytes best discriminated RM and control populations. Women with RM and high NK levels may have an immunological disorder.

Key words: recurrent miscarriage / natural killer cells / early pregnancy loss / flow cytometry / immunology of pregnancy

Introduction

Recurrent miscarriage (RM), defined as three or more consecutive pregnancy losses prior to 20 weeks, occurs in 1–3% of women (Choudhury and Knapp, 2000; Carrington et al., 2005). Up to half of these cases remain unexplained by routine RM screening (Druckmann and Druckmann, 2005). RM causes significant psychosocial morbidity, and for many couples the label of ‘unexplained’ is unacceptable.

The immune system has long been implicated in miscarriage. Medawar (1953) proposed that pregnancy involved immune modulation to protect a fetal semi-allograft from rejection, and much subsequent work defined a role for altered maternal lymphocyte profiles and trophoblast MHC expression (Sacks et al., 1999). Wegmann et al. (1993), in a landmark paper, changed our view of maternal immunology by demonstrating that the immune system was not universally suppressed, but rather shifted to favour type 2 (antibody-mediated) over type 1 (cell-mediated) responses. It was then a simple extrapolation that an aberrant type 1 response may cause miscarriage (Raghupathy, 1997). While potential mechanisms have been described for achieving the type 2 shift for normal pregnancy involving progesterone and progesterone-induced binding factor (Roth et al., 1996; Szereres-Barthos and Wegmann, 1996), and the tryptophan catabolizer IDO (Munn and Mellor, 1998), it has also been recognized that maternal immune modulation is more complex (Germain et al., 2007; Chaouat, 2008). There has been renewed interest in previous observations that certain elements of the maternal innate immune system are activated in normal pregnancy (Chaouat et al., 1999; Sacks et al., 1999; Sacks et al., 2003; Germain et al., 2007). Thus, it is of interest that maternal innate natural killer (NK) cells are strikingly suppressed in normal early pregnancy (Szereres-Barthos and Wegmann, 1996; Sacks et al., 2003). It is hypothesized that NK cells that are not suppressed (or are indeed...
activated) could cause a type I shift and miscarriage in some women (Chaouat, 2008).

NK cells are innate lymphocytes with a CD3−CD56+ phenotype. The CD56+Bright NK subset is CD16+, has a high IL-2 affinity and produce cytokines. The CD56−Dull subset is CD16+, has moderate IL-2 affinity and orchestrate NK cytotoxicity (Cooper et al., 2001). These subsets also express different monocyte-derived cytokine and MHC-recognizing receptors including the activating receptor CD69 (Cooper et al., 2001; Coulam and Roussev, 2003). NK cells are present in the peripheral blood and in the uterine tissue, where they appear to regulate trophoblast invasion (Moffett-King, 2002; Moffett et al., 2004). NK cells are the dominant uterine immune cell in pregnancy (Vince and Johnson, 2000; Koopman et al., 2003), although their activity appears to be suppressed (Szereres-Barthos and Wegmann, 1996). These cells are predominantly CD56+Bright, whereas this subset represents only 10% of the peripheral blood population, and the relationship between peripheral and uterine NK cells is unclear (Rai et al., 2005).

Several studies have examined the link between altered peripheral NK levels and RM. Women with RM have been reported to have high NK cytotoxicity measured by Cr51 releasing assay (Aoki et al., 1995; Shakhar et al., 2003). Increased NK numbers were first reported by Beer et al. (1996) as a percentage of total peripheral lymphocytes. This study of 81 women with RM and 17 controls proposed that NK levels >18% should be considered extremely high. Little statistical detail was given to support their conclusion. Others have found an association between this parameter and primary RM only (Shakhar et al., 2003). Few papers have specifically reported on the relationship between the CD56−Dull NK subset and RM (Beer et al., 1996; Emmer et al., 2000.), with no studies demonstrating a significant difference in this parameter between RM and control women. Similarly, there is little information on the relationship between the activation marker CD69 and RM. A small study (n = 40 cases, n = 13 controls) reported that NK cells from women with RM stimulated in vitro expressed more CD69 than NK cells from controls (Ntrivalas et al., 2001). And a recent unconfirmed report has shown that NK cells from women with RM do appear to have increased CD69 expression (Prado-Drayer et al., 2008).

Peripheral NK cell analysis is further confounded as the normal NK range for women of reproductive age is unclear. Bisset et al. (2004) reported a large physiological NK range for females (5.33–20.25% of total peripheral lymphocytes); however, this study did not exclude women with reproductive problems. Additionally, biopsychosocial variables may influence NK levels. Men have higher levels than women (Giglio et al., 1994; Yovel et al., 2001), and NK parameters can be transiently raised by acute stress and exercise (Goebel and Mills, 2000; Hosaka et al., 2002; Bouillon et al., 2006; Shakhar et al., 2006), and women undergoing ovarian stimulation for IVF (Giuliani et al., 1998).

There is understandable concern that the day of the menstrual cycle may also have an effect, although data are conflicting. NK numbers have been reported to be higher in the luteal phase (Yovel et al., 2001) or unchanged throughout the cycle (Giuliani et al., 1998), and NK cytotoxicity has been reported to be lower in the luteal phase (Souza et al., 2001) or unchanged (Yovel et al., 2001). In any case, this variable needs to be controlled to ensure consistency of results.

This study aimed to determine whether there was a real difference in preconceptual peripheral NK parameters between women with a history of RM and healthy control women, to ascertain which parameters best differentiated these two cohorts, and to determine what NK levels should be considered high.

Materials and Methods
About 104 non-pregnant women with a history of three or more consecutive miscarriages (61 nulliparous and 43 parous women) were compared with 33 healthy control women (14 nulliparous and 19 parous women). For the sake of consistency and to coincide with other tests, all blood samples were taken in the mid-luteal phase. Clinical data from both cohorts were obtained retrospectively to conduct a baseline analysis for the variables of patient age, number of pregnancies, number of consecutive miscarriages, number of live births, body mass index, past medical and surgical history and medications. The RM cohort was then further subdivided into RM+ women who tested positive to one or more RM screening tests, and RM− women who tested negative to all screening tests or who had been adequately treated for an abnormality and continued to miscarry. These tests included male and female karyotype, hormone tests (FSH, LH, testosterone, SHBG), diabetes screen (insulin, BSL, HbA1c), comprehensive thrombophilia screen (FBC, protein-S, protein-C, AT3, activated protein-C resistance, prothrombin gene mutation, factor V leiden, anticardiolipin antibodies, lupus inhibitor, MTHFR mutation), sperm tests (DNA fragmentation) and anatomical tests (ultrasound and HyCoSy/hysterosalpingogram/hysteroscopy). It was hypothesized that couples in the RM+ group would be particularly frustrated with their diagnosis of unexplained RM, and might benefit from further investigation.

Prior to staining for surface marker analysis, a white cell count and lymphocyte percentage was obtained from the Sysmex XE2100 Haematology analyser (Roche Diagnostics). Surface marker analysis on peripheral blood samples was performed using direct immunofluorescence and a lyse/wash protocol and was performed within 4 h of collection. Four tubes were set up per patient using the monoclonal antibodies to CD45-PerCP, CD3-APC, CD19-APC, CD56-PE, CD69-FITC (Becton Dickinson, San Jose, CA), CD14-PE and CD16-FITC (Immunotech, Marseilles, France). Isotype and fluorochrome matched controls (Becton Dickinson) were used as negative controls. Antibodies were used at the volumes recommended by the manufacturer and incubated for 10 min with a volume of peripheral blood to give a concentration of 1 x 106 white cells per tube. Cells were then lysed and fixed with FACS lysing solution (Becton Dickinson) for 15 min before being washed three times prior to analysis. Flow cytometric analysis was performed on the Becton Dickinson FACSCalibur dual laser flow cytometer (488/633 nm) using CellQuest software (Becton Dickinson). For NK subset determination, 2000 NK cells (CD3 negative, CD56 positive) were collected, and 10 000 NK cells for measurement of activation using CD69. Total NK cells and the CD56−Dull subset were expressed as a percentage of lymphocytes as well as absolute number. The activated CD69+CD56−Dull subset was expressed as a percentage of CD56−Dull cells as well as an absolute number (Fig. 1).

Results were analysed with GraphPad Prism software (San Diego, CA). Mann–Whitney and Fisher’s exact tests were used to compare continuous and dichotomous variables, respectively.

Results
Women in the RM cohort were aged 25–49 (mean = 36.9) and experienced on average 5.34 pregnancies (range = 5–17) and 4.4 miscarriages (range = 3–15), conceived either naturally or through IVF. Controls were aged 20–47 (mean = 34.4), had experienced two or more miscarriages (mean = 0.303), and had an average of
1.18 live births. There was no significant difference between RM and control cohorts for the variables of age, BMI, number of cigarettes currently smoked per day or units of alcohol consumed per week. No subject in either group took excessive alcohol, and there were few smokers in either cohort. Comparison of past medical and surgical histories revealed a higher prevalence of known autoimmune disease prior to testing in women with RM compared with controls. These included the presence of Graves’ disease (n = 3), Hashimoto’s disease (n = 3), SLE and antiphospholipid antibodies (n = 4), scleroderma (n = 3), psoriasis (n = 2) and Sjogren’s syndrome (n = 1).

The comparison of NK parameters between women with RM and controls is shown in Table I. NK cells as a percentage of total peripheral lymphocytes was significantly elevated in the RM population (P = 0.0004), and the CD56$^{+}$Bright:CD56$^{+}$Dim ratio was significantly lower (P = 0.0365). In the RM group, the increased mean levels of NK cell concentration, CD56$^{+}$Dim NK cell concentration and percentage and CD56$^{+}$DimCD69$^{+}$ NK concentration did not reach statistical significance.

A ROC analysis (AUC = 0.706) comparing NK percentage between the RM and control cohorts was performed (Fig. 2). NK percentage >18% differentiated the cohorts with a sensitivity of 12.5 (95%CI = 6.83–20.4), specificity of 97.0 (95%CI = 84.2–99.9) and likelihood ratio of 4.12.

From the whole RM cohort, women who had had one or more previous live births were compared with women who had no live births. There was no statistically significant difference in the NK percentage between women who had had no live births (mean 12.4%) compared with those who had (10.6%, P = 0.119).
A subgroup of 46 women with RM who were screening test positive was then compared with controls (Table II). That subgroup had a significantly higher NK percentage ($P = 0.0008$), CD56$^+$ Dim NK percentage ($P = 0.0375$) and CD56$^+$ Dim CD69$^+$ concentration ($P = 0.0209$). CD56$^+$ Bright/CD56$^+$ Dim ratio was also significantly lower ($P = 0.0216$) and no other variables differed significantly between groups. Profiling of RM screening test results revealed a high anticardiolipin antibody (ACA) prevalence in this group (40.9%). In a subanalysis, women with ACAs were compared with women without ACAs and controls. NK percentage and concentration were significantly higher in those with ACAs compared with those without ACAs ($P = 0.0417$ and $P = 0.0477$, respectively). Women with RM and ACAs had significantly higher NK percentage and concentration, CD56$^+$ Dim NK percentage and concentration and CD56$^+$ Dim CD69$^+$ concentration. CD56$^+$ Bright/CD56$^+$ Dim ratio was significantly lower. NK percentage was the only variable that was significantly higher in the ACA-negative RM group compared with controls ($P = 0.002$).

Figure 2 ROC analysis of NK percentage in the RM cohort compared with controls.

**Discussion**

This study supports previous reports that women with RM have altered peripheral blood NK parameters (increased numbers and/or levels of activation) compared with control women without a history of RM (Ntrivalas et al., 2001; Beer et al., 1996; Shakhar et al., 2003). Moreover, women in the RM$^-$ cohort, who are perhaps most frustrated by having no cause found for RM, had significantly raised NK percentage. Emmer et al. (2000) failed to detect a significant difference in preconceptual NK percentage between women with RM and controls, possibly due to the broader analysis of including women with two or more miscarriages (rather than more than three in this study). Our study found that using NK percentage to define high NK levels identified women with RM with a very high specificity in this sample space, supporting Beer et al. (1996). Furthermore, the low test sensitivity supports others (Beer et al., 1996; Emmer et al., 2000; Shakhar et al., 2003) in demonstrating, reasonably, that many women with RM do not have elevated NK levels. This means that NK testing would be an ineffective way to identify women with RM from the general population, but is able to effectively identify a subpopulation of women with known RM who may benefit from immunosuppressive therapy.

Although NK percentage was the most significant difference between women with RM and controls, an overall view of all parameters analysed (including NK concentration, CD56$^+$ Dim concentration and percentage, CD56$^+$ Dim/CD56$^+$ Bright ratio and CD56$^+$ Dim expression of the activation marker CD69) was consistent with a shift in the peripheral blood NK activity in RM as has been previously reported (Beer et al., 1996; Emmer et al., 2000; Ntrivalas et al., 2001). Together, these data indicate that a peripheral shift in the NK subtypes and activation might be an indication of a genuine mechanism for immune dysfunction causing miscarriage. It is commonly argued that blood and uterine NK...
cells are different phenotypes, and that uterine NK cells (CD56^Bright) are benign, cytokine producing and probably essential for normal pregnancy (Moffett-King, 2002). However, it is now also accepted that uterine NK cells are different phenotypes, and that uterine NK cells (CD56^Bright) results in increased recruitment of CD56^Bright cells, increased activated peripheral blood NK cells. It is well established that the mechanism for miscarriage in ACA positive women does not appear to be solely thrombotic (Di Simone et al., 2007). There is evidence of direct toxic effects of ACAs on trophoblast cells, and also immune dysregulation. We propose that increased NK activity in those women provides a further potential immune mechanism for poor placentation and miscarriage. Larger studies are required to assess this association, but its importance is clear from a clinical perspective. Currently women with RM and ACAs are treated with heparin and aspirin. Those with increased NK cell activity might also benefit from immune suppressive therapy (e.g. prednisone).

The use of immune suppressive therapy in women with RM has a long and controversial history. A review has concluded that there is no proven benefit in women with otherwise unexplained RM taking intravenous immunoglobulin (IVIG) or leucocyte infusions (LIT) (Porter et al., 2006). However several small studies suggest that these therapies may benefit subsets of RM patients with immunological abnormalities. In particular, IVIG and LIT have been shown to reduce NK levels (Szpakowski et al., 2001; Perricone et al., 2006) or cytotoxicity (Kotlan et al., 2008), and has been reported to suppress NK cell activity (Thum et al., 2008), and has been reported to be effective in women with RM in anecdotal reports (Quenby et al., 2006). Prednisolone is a further treatment option that can be effective in women with RM and raised ACAs (Laskin et al., 1997), but there has not yet been a trial using prednisolone in women with high NK cell levels. It should be noted that all three potential therapies (IVIG, LIT and prednisolone) are used for their non-specific immune suppressive effects, but prednisolone has significant potential advantages. It is cheaper, easier to take, does not require blood screening and most clinicians are familiar with it. In view of potential side effects on mother and

### Table II Summary of NK parameter alterations and significance in the RM⁺ and RM⁻ cohorts compared with controls

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Variables</th>
<th>NK%</th>
<th>NK Conc</th>
<th>Dim NK%</th>
<th>Dim NK Conc</th>
<th>Bright:dim</th>
<th>CD69 Dim NK%</th>
<th>CD69 Dim NK Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>n</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>8.80</td>
<td>0.200</td>
<td>92.5</td>
<td>0.189</td>
<td>0.0912</td>
<td>2.62</td>
<td>0.00418</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>5.75</td>
<td>0.17</td>
<td>94.3</td>
<td>0.16</td>
<td>0.06</td>
<td>2.29</td>
<td>0.0037</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>3.53–34.0</td>
<td>0.01–0.73</td>
<td>69.7–99.7</td>
<td>0.05–0.73</td>
<td>0.01–0.45</td>
<td>0.58–8.93</td>
<td>0.00097–0.0138</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>5.37</td>
<td>0.129</td>
<td>6.41</td>
<td>0.130</td>
<td>0.0886</td>
<td>1.67</td>
<td>0.00262</td>
</tr>
<tr>
<td>RM⁺ Cohort</td>
<td>n</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>11.9</td>
<td>0.237</td>
<td>95.2</td>
<td>0.229</td>
<td>0.344</td>
<td>2.75</td>
<td>0.00616</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>10.8</td>
<td>0.225</td>
<td>96.6</td>
<td>0.215</td>
<td>0.0400</td>
<td>2.47</td>
<td>0.00509</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>4.17–27.5</td>
<td>0.06–0.7</td>
<td>83.0–98.7</td>
<td>0.05–0.69</td>
<td>0.01–0.21</td>
<td>0.76–6.13</td>
<td>0.00135–0.0154</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>5.38</td>
<td>0.128</td>
<td>3.50</td>
<td>0.128</td>
<td>0.0398</td>
<td>1.18</td>
<td>0.00390</td>
</tr>
<tr>
<td>P (Mann–Whitney) RM⁺ versus controls</td>
<td>0.9069</td>
<td>0.0375</td>
<td>0.0736</td>
<td>0.0216</td>
<td>0.311</td>
<td>0.0209</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM⁻ cohort</td>
<td>n</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>11.1</td>
<td>0.225</td>
<td>94.3</td>
<td>2.15</td>
<td>0.0652</td>
<td>2.78</td>
<td>0.00471</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>9.96</td>
<td>0.251</td>
<td>95.2</td>
<td>0.185</td>
<td>0.05</td>
<td>2.33</td>
<td>0.00397</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>3.08–22.0</td>
<td>0.06–0.54</td>
<td>81.2–99.1</td>
<td>0.050–0.530</td>
<td>0.01–0.22</td>
<td>0.65–8.38</td>
<td>0.00144–0.0125</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>4.43</td>
<td>0.108</td>
<td>4.09</td>
<td>1.084</td>
<td>0.0595</td>
<td>1.68</td>
<td>0.00241</td>
</tr>
<tr>
<td>P (Mann–Whitney) RM⁻ versus controls</td>
<td>0.138</td>
<td>0.188</td>
<td>0.107</td>
<td>0.129</td>
<td>0.713</td>
<td>0.225</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total NK percentage (NK%) = NK concentration/total peripheral lymphocytes × 100. Total NK concentration (NK conc) = NK count × 10⁹/l. CD56^Dim NK cell percentage (% Dim NK) = CD56^Bright NK concentration/NK concentration × 100. CD56^Dim NK concentration (Dim NK Conc) = CD56^Bright NK count × 10⁹/l. CD56^Bright/CD56^Dim ratio (Bright:dim) = CD56^Bright NK concentration/CD56^Dim concentration. Percentage of activated (CD69^Bright) CD56^Dim NK cells (%CD69 Dim) = CD69^Bright CD56^Dim concentration/CD56^Dim concentration × 100.
fetus (Empson et al., 2005), immune therapy certainly requires caution. However, we believe that this study provides a firm rationale for large-scale clinical trials on the effect of immunosuppression in women with RM and high peripheral blood NK cells.

It is not known what link, if any, exists between uterine and peripheral blood NK cells. It is accepted that uterine NK cells appear to play an important role in the early implantation, but whether aberrations cause miscarriage is unknown. In any case, this study and others demonstrate an independent association between peripheral NK cells and RM. This is an important observation, as much debate previously has revolved around the significant differences between peripheral blood and uterine NK cells (Moffett et al., 2004) with the underlying assumption that NK cells themselves are hypothesized to be direct causes of miscarriage. We have presented a pilot study that demonstrated a strong correlation between blood and uterine NK cells (Fay et al., 2007), particularly when levels were high. If this finding was confirmed, it is possible to propose a mechanism whereby increased numbers, increased cytotoxic CD56^+Dim subtypes and increased activated cells (expressing CD69) in the blood leads to increased levels of such cells recruited into the uterus every month, producing a hostile uterine environment for implantation. However, we also propose an alternative hypothesis. The immune system is complex and works as a systemic network. It is highly unlikely that a single cell type is the sole cause of miscarriage in those women. Raised NK cell activity has not been recognized in any other disease state that we are aware of, apart from abdominal aortic aneurysm (Forester et al., 2006). Thus perhaps we should interpret NK activity as just one measure of overall immune function. It has been proposed that, more accurately, there may be a syndrome in which the presence of various immune factors (NK cells in blood, NK cells in uterus, ACAs, thyroid antibodies, etc.) increase the likelihood of an immune reproductive disorder (Gleicher, 2002).

In conclusion, this study is one of the largest and most detailed flow cytometric analyses of preconceptual peripheral blood NK cells in women with RM. It clearly demonstrated that women with RM have significantly increased NK activity, with NK percentage being the parameter that best differentiated test and control groups. By a simple blood test, 12.5% of women with RM were found to have an NK percentage >18% compared with only 3% of the control population. It is not yet proven that high NK levels signal a pathological mechanism predicting miscarriage. Nor is it known how NK levels come to be raised, how long they remain high, or what long-term health consequences might be. Nevertheless, we believe that randomized controlled studies are indicated to assess whether women with such high NK levels would benefit from immune therapy.

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