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Peripheral natural killer cytotoxicity and $CD56^{pos}CD16^{pos}$ 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 $CD56^{pos}$ and double $CD56^{pos}CD16^{pos}$ 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 $CD56^{pos}$ cells, but cytotoxic function appeared unaffected. The expression pattern of killer inhibitory receptors on $CD56^{pos}$ cells was not different between patients and controls. A longitudinal study revealed that, compared with controls, in RSA women higher numbers of double $CD56^{pos}CD16^{pos}$ cells were present during early pregnancy, paralleled by an increase in cytotoxic NK cell reactivity. The single $CD56^{pos}$ population decreased in number. In conclusion, the analysis of peripheral NK cell characteristics appears a suitable diagnostic tool in RSA. Immunomodulation 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 cytotoxity) 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 preventive measures, e.g. i.v. injection of immunoglobulin (Ig)G (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, anticardiolipin 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, 100U/ml penicillin and 100 µmol/l streptomycin (both Life Technologies); 1×10^6 K562 target cells were labelled with 3.7 MBq ^{51}Cr (Amersham, UK) for 2 h. Then, 1×10^3 labelled target cells were added to 80×10^3, 40×10^3, 20×10^3 or 10×10^3 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 µl of supernatant was harvested and radioactivity was measured using a γ-counter (Wallac 1470 γ counter, Turku, Finland). The percentage specific NK cell cytotoxic reactivity was determined as follows:

$$\%\ \text{lysis} = \frac{\text{sample value–spontaneous release}}{\text{maximum release–spontaneous release}}$$

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 10^7 effector cells (LU = 10^7/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-Epics (Coulter Electronics) and 5000 or 10 000 events were collected based on live lymphocyte cell gating as indicated by propidium iodide (5 µg/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 αCD16-FITC (clone DJ130c), αCD56-RPE (clone MOC-1), αCD3-FITC or αCD3-RPE (clone UCHT1) antibodies were used (all from Dako, Glostrup, Denmark). NK cell inhibiting/activating receptor expression was characterized by conjugated αCD158a-PE (clone EB6), αCD158b-PE (clone GL183) and unconjugated αNKG2a (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

Table I. The mean and 95% confidence interval (CI) of pre-conceptional natural killer (NK) cell cytotoxicity (lytic units) and percentage of NK cell subsets in recurrent spontaneous abortion (RSA) women and controls, before and during folic acid treatment

	Folic acid	Intake controls Mean (CI)	RSA Mean (CI)	Follow up controls Mean (CI)	P^a	P^b	P^c
Lytic units	before	445.0 (415.6; 475.9)	411.0 (378.1; 444.0)	–	NS	NS	NS
	during	–	393.1 (362.2; 423.9)	364.5 (338.1; 391.0)			
% $CD56^{pos}CD3^{neg}$	before	13.5 (10.8; 16.3)	13.2 (10.4; 16.0)	–	NS	NS	NS
	during	–	15.8 (12.8; 18.8)	12.5 (8.8; 16.2)			
% $CD56^{pos}CD16^{pos}CD3^{neg}$	before	12.0 (9.6; 14.4)	11.6 (9.0; 14.2)	–	NS	NS	NS
	during	–	12.9 (10.1; 15.7)	11.7 (8.4; 15.0)			
% $CD56^{pos}CD16^{neg}CD3^{neg}$	before	1.4 (0.7; 2.1)	1.4 (0.7; 2.2)	–	NS	NS	0.01 to <0.05
	during	–	2.4	(1.5; 3.4)	1.8 (0.7; 3.0)		
% $CD56^{pos}CD16^{neg}/CD56^{pos}$ $CD16^{pos}$	before	0.39 (0.21; 0.57)	0.25 (0.06; 0.44)	–	NS	NS	<0.01
	during	–	0.55 (0.28; 0.82)	0.56 (0.23; 0.89)			

NS = not significant.
[a]*P* value for the difference between mean value in the RSA women and the intake controls, using the *t*-test for independent samples.
[b]*P* value for the difference between mean value in the RSA women and the follow-up controls.
[c]*P* value for the difference between mean value in the RSA women before and after folic acid treatment.

significance of the differences in the mean values of age and NK cell parameters within the RSA group before and after folic acid supplementation. The mean values with 95% CI are presented.

Repeated measurement analysis adjusting for the pre-conceptional value was used to test for statistical significance of the differences between the RSA group and follow-up controls in the mean NK cell parameters at 8 and 12 weeks after conception. The estimated means with 95% CI are presented.

Differences between the groups may be influenced by the loss of information of those women who miscarried. Therefore the repeated measurement analysis was also performed using the method of last observation carried forward (LOCF). The LOCF method was performed at each assessment point, substituting missing values after miscarriage at all subsequent assessments by the last observed value after conception. The results of both analyses were similar and those using the LOCF method are presented here. It is obvious that these variables are not mutually independent. Therefore the *P* values are presented unadjusted for analysing multiple variables. Note that *P* values are adjusted for multiple comparisons of each variable (Table II: Tukey–Kramer).

Finally, the influence of the pre-conceptional value of age and NK-cell parameters, separately, on the probability of miscarriage in women with RSA was analysed using univariate logistic regression. The crude odds ratios with 95% CI are presented. The dichotomized data were analysed using Fisher's exact test.

Results

Pre-conceptional peripheral NK cell reactivity and cell numbers

We assessed whether in our centre pre-conceptional peripheral cytotoxic NK cell reactivity, NK and T cell levels in women with a history of unexplained recurrent spontaneous miscarriages differed from controls. Table I shows the estimated values before and during folic acid treatment for NK cell cytotoxicity (LU), peripheral NK cell and $CD3^+$ T cell levels.

Before folic acid treatment analysis a mean LU level of 411 (95% CI: 378.1; 444.0) was found in RSA women, which was not significantly different from the level found in intake controls (mean 445.0, 95% CI: 415.6; 475.9) ($P = 0.126$). Similarly, no difference was found for either $CD56^{pos}CD3^{neg}$ NK or $CD3^{pos}$ T cell levels between RSA women before folic acid treatment and controls. In both groups the percentage of cells was ~13% for NK cells and 68% for T cells. Analysis of subset division of NK cells into double $CD56^{pos}$ $CD16^{pos}$ and single $CD56^{pos}$ cells did not reveal a statistically significant difference between the groups.

During folic acid treatment NK cell levels in RSA women were compared with follow-up control women, successfully partaking in an IVF procedure and also receiving folic acid treatment. None of the parameters tested revealed a substantial difference between RSA women and controls. The estimated mean LU levels in the RSA and follow-up control group were 393.1 (95% CI: 362.2; 423.9) and 364.5 (95% CI: 338.1; 391.0) respectively ($P = 0.165$) and the mean total NK cell levels were 15.8% (95% CI: 12.8; 18.8) and 12.5% (95% CI: 8.8; 16.2) respectively ($P = 0.169$).

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; 44.0) 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 $CD56^{pos}$ 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 $CD56^{pos}$/double $CD56^{pos}CD16^{pos}$ 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

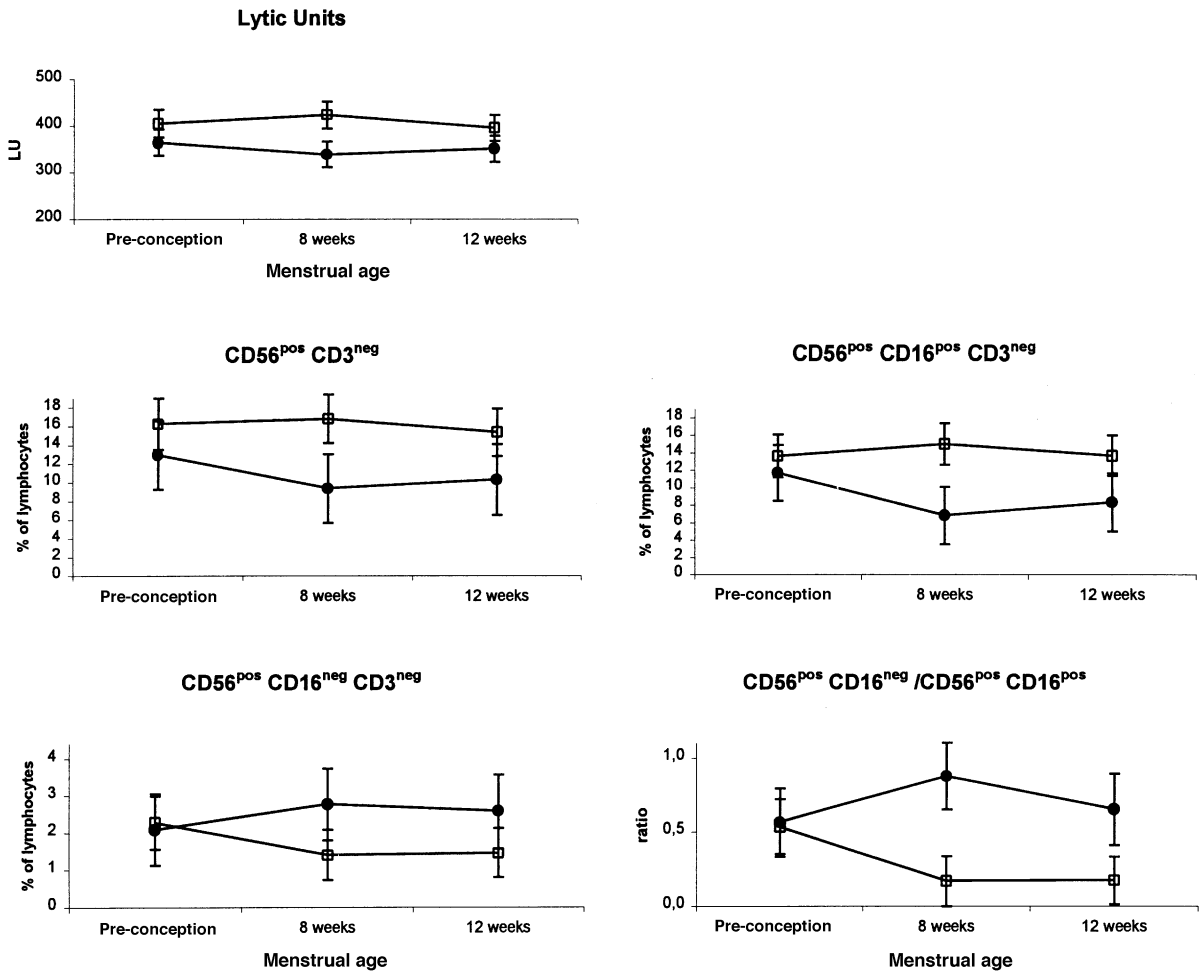

Figure 1. The estimated mean natural killer (NK) cell cytotoxic reactivity (LU) and NK cell parameters in recurrent spontaneous abortion (RSA) women (□) and follow-up controls (●) at each assessment point, using the method of last observation carried forward (LOCF). The vertical lines indicate the 95% 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 $CD56^{pos}CD16^{pos}$ 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 $CD56^{pos}$ cells were lower in RSA women as compared with controls, both at weeks 8 and 12. In combination with the higher levels of double $CD56^{pos}CD16^{pos}$ cells this resulted in a notable reduction in the ratio single $CD56^{pos}$/double $CD56^{pos}CD16^{pos}$ NK cells [–0.65 (95% CI: –1.0; –0.3)]. Notably, the concomitant rise of NK cell cytotoxicity, increased numbers of $CD16^{pos}$ NK cells and decreased levels of single $CD56^{pos}$ cells fits the known characteristics of NK cell activation.

$CD3^{pos}$ cell numbers (including the $CD3^{pos}CD56^{pos}$ 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 $CD56^{pos}CD16^{pos}$ cells, % single $CD56^{pos}$ cells, ratio single $CD56^{pos}$/double $CD56^{pos}CD16^{pos}$

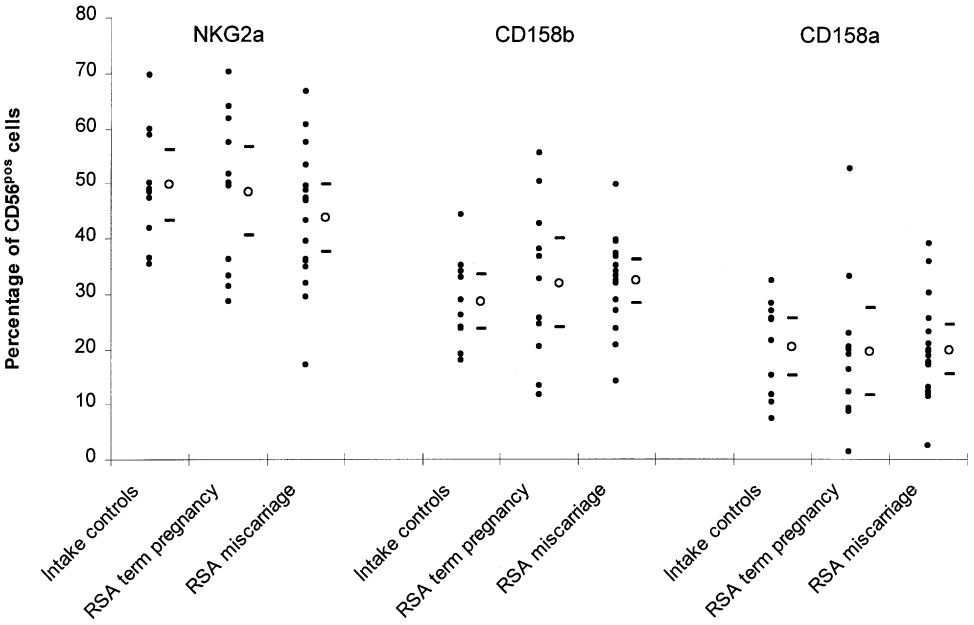

Figure 2. Expression of CD158a and CD158b (with specificity for HLA-C molecules) and expression of NKG2a receptor (specific for HLA-E, complexed with, amongst others, processed HLA-G molecules) on $CD56^{pos}$ cells. Depicted are individual readings (●), means (○) and 95% confidence interval (–).

Table II. Estimated differences in mean natural killer (NK) cell parameters between recurrent spontaneous abortion (RSA) women and follow-up controls during pregnancy measured at both week 8 and 12 of menstrual age using repeated measurement analysis adjusted for the pre-conceptional value and on the basis of last observation carried forward

	Mean difference (CI) between RSA women and follow-up controls			Simultaneous levels of significance			
	Overall	Week 8 menstrual age	Week 12 menstrual age	P^a	P^b	P^c	P^d
Lytic units	31.0 (2.5; 59.6)	55.2 (10.6; 99.8)	6.9 (–37.1; 50.9)	0.01 to <0.05	NS	0.01 to <0.05	<0.01
% $CD56^{pos}$ $CD3^{neg}$	4.0 (0.4; 7.6)	5.4 (–0.2; 11.0)	2.6 (–2.9; 8.1)	0.01 to <0.05	NS	NS	<0.01
% $CD56^{pos}CD16^{pos}CD3^{neg}$	4.9 (2.0; 7.8)	6.4 (1.8; 10.9)	3.4 (–1.1; 7.9)	<0.01	NS	NS	<0.01
% $CD56^{pos}CD16^{neg}$ $CD3^{neg}$	–1.0 (–1.8; –0.2)	–1.0 (–2.2; 0.2)	–1.0 (–2.2; 0.3)	0.01 to <0.05	NS	NS	<0.01
% $CD56^{pos}CD16^{neg}/CD56^{pos}CD16^{pos}$	–0.55 (–0.75; –0.36)	–0.65 (–1.0; -0.30)	–0.45 (–0.81; –0.08)	<0.01	NS	NS	NS

NS = not significant.
[a]Adjusted *P* value according to the method of Tukey–Kramer of the overall difference between RSA women and follow-up control women.
[b]*P* value of the test for statistical significance between the assessment points.
[c]*P* value of the test for statistical significance of the group differences between the assessment points.
[d]*P* value of the test for statistical significance of the regression of the pre-conceptional value during folic acid treatment, indicating that the pre-conceptional value significantly contributes to estimate the parameter at each assessment point.

cells, % $CD3^{pos}$ cells, % double $CD3^{pos}$ $CD56^{pos}$ 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 effect in either group.

KIR/KAR and NKG2a expression on $CD56^{pos}$ 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

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

Parameter	Threshold value	Successful pregnancy[a]	Miscarriage[a]	*P* value
Lytic units	<322	4 (3)	3 (3)	
	⩾322	12 (7)	5 (3)	NS
% $CD56^{pos}CD3^{neg}$	<12.0	8 (5)	0 (0)	
	⩾12.0	7 (4)	7 (5)	⩽0.01 (0.05 to <0.10)
% $CD56^{pos}CD16^{pos}CD3^{neg}$	<20.7	12 (8)	7 (6)	
	⩾20.7	4 (2)	2 (1)	NS
% $CD56^{pos}CD16^{neg}CD3^{neg}$	<4.0	14 (8)	5 (3)	
	⩾4.0	1 (1)	2 (3)	NS
% $CD56^{pos}CD16^{neg}/CD56^{pos}$ $CD16^{pos}$	<0.08	5 (3)	2 (1)	
	⩾0.08	10 (6)	5 (5)	NS

NS = not significant.
[a]Number of RSA women with successful pregnancy or subsequent miscarriage after dichitomizing using the specific threshold values. Values in parentheses are the numbers of RSA women with three or more subsequent miscarriages.

2). Expression was similar for all groups studied. CD158a, CD158b and NKG2a were expressed on approximately 20, 30 and 45% of $CD56^{pos}$ 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 $CD56^{pos}$ 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 $CD56^{pos}CD16^{pos}$ 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 found in the controls. The controls were women partaking in an IVF procedure, but exactly the same trend was previously observed for other healthy pregnant women (Okamura *et al.*, 1984), validating our choice of controls in this respect.

In the RSA women the ratio of single $CD56^{pos}$/double $CD56^{pos}CD16^{pos}$ cells was altered in favour of the CD16-expressing cells. Since CD16, and not CD56, was recently characterized as a lysis receptor mediating direct cytotoxicity (Mandelboim *et al.*, 1999), the concomitant rise in levels of cytotoxic reactivity found in the RSA group was not surprising.

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. As in our study, both groups used as definition two or more miscarriages; however, differences in protocols, for instance the influence of cryopreserved versus fresh PBMC (Kawai *et al.*, 1988), cannot be excluded. 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 $CD56^{pos}CD16^{pos}$ cells and a concurrent drop in single $CD56^{pos}$ 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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