Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss?

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BACKGROUND: Successful pregnancy may depend on a Th2-type cytokine response, whilst, conversely, a poor pregnancy outcome may be associated with an increase in Th1 cytokines and a concomitant decrease in Th2 cytokines. This prospective study was designed to elucidate whether a failure of the cytokine shift pre-dated miscarriage and was therefore likely to be an aetiological factor in recurrent pregnancy loss (RPL). METHODS: Cytokine production by stimulated peripheral blood mononuclear cells from 46 pregnant women who had previously suffered idiopathic RPL during early pregnancy was compared with 25 gestationally age-matched pregnant controls and 11 non-pregnant women. RESULTS: Production of IFN-γ was lower in pregnant than in non-pregnant women and even lower in RPL pregnant women ($P = 0.0191$). IL-10 was increased in pregnant women compared with non-pregnant controls, and further increased in RPL patients ($P = 0.026$). IL-4 was also increased in women with RPL ($P = 0.0001$). No differences in IFN-γ, IL-10 or IL-4 secretion were observed in RPL patients who subsequently miscarried compared with those who successfully completed the pregnancy. RPL women with a successful reproductive outcome had similar concentrations of TNF-α to pregnant women, RPL women who subsequently miscarried had significantly lower levels than either pregnant women ($P = 0.02$) or non-pregnant controls ($P = 0.0004$). CONCLUSIONS: Contrary to our hypothesis, the cytokine shift, which appears to characterize normal pregnancy, was accentuated rather than diminished in RPL pregnant women.

Key words: cytokines/peripheral blood monocytes/pregnancy outcome/recurrent pregnancy loss

Introduction

Recent attention has focused on elucidating the immunobiological roles of cytokines in normal human pregnancy following the accumulated reports of complex cytokine activity within uteroplacental tissues (Robertson et al., 1994; for review). T helper (Th) cells can differentiate into subsets with distinctive patterns of cytokine release. It has been proposed that Th1-type responses (e.g. the production of IL-2 and IFN-γ) are systemically suppressed in murine pregnancy and that local expression of Th2-type cytokines (e.g. IL-4, IL-6, IL-10) in placental tissue might be beneficial for fetal survival (Wegmann et al., 1993). Whether an analogous situation exists in human pregnancy is unclear as yet (Vince and Johnson, 1996), although partial systemic impairment of Th1 responses is compatible with clinical evidence that a number of infectious diseases caused by intracellular pathogens can sometimes be exacerbated in pregnancy, e.g. cytomegalovirus and malaria (Hart, 1988). Furthermore, rheumatoid arthritis, characterized by a Th1 response, often undergoes remission during pregnancy (Da Silva and Spector, 1992).

Successful pregnancy may depend, at least in part, on the bias of the maternal immune response shifting away from Th1 type responses towards a Th2 phenotype, both in murine models and humans. An abnormal Th1-type cellular immune response is the basis for a recent hypothesis for immunological reproductive failure in women (Hill et al., 1995). Peripheral blood mononuclear cells (PBMC) from women with recurrent pregnancy loss (RPL) respond to trophoblast extracts in vitro by: (i) releasing soluble factors that adversely affect embryo and trophoblast viability; (ii) releasing Th1-type cytokines (TNF-α and IFN-γ) and (iii) a reduction in Th2-type cytokine production (IL-4 and IL-10) (Hill et al., 1992; Ecker et al., 1993; Yamada et al., 1994; Hill et al., 1995; Raghupathy et al., 1999). In contrast, PBMC from women with a history of successful pregnancies produce Th2-type cytokines when exposed to trophoblast extracts (Hill et al., 1995; Raghupathy et al., 1999). Circulating levels of TNF-α and IFN-γ are higher in patients with a subsequent miscarriage compared with those with a successful pregnancy, suggesting that these cytokines may also be a potentially relevant factor in RPL patients (Müller-Eckhardt et al., 1994; Jenkins et al., 2000). In mouse models of RPL, the presence of Th2-type cytokines such as IL-4, IL-5, IL-6 and IL-10 appears to be associated with successful pregnancy, whilst Th1-type cytokines such as...
IFN-γ and IL-2 are associated with embryo demise (Chauvat et al., 1990).

Marzi et al. examined cytokine production by PBMC obtained from women throughout pregnancy (Marzi et al., 1996). Normal pregnancy was accompanied by a decrease in Th1 (IL-2 and IFN-γ) productive capacity together with an increase in Th2 (IL-4 and IL-10) production, most notably in the third trimester. In five women who subsequently miscarried (a single miscarriage and not RPL), there was an increased production of Th1 cytokines (IL-2) whereas Th2 cytokines (IL-10) were reduced. A study of five women who delivered small-for-gestational-age babies demonstrated a reduced capacity for IL-10 production by PBMC, in contrast to a marked increase observed in amniotic fluid IL-10 in intrauterine growth retardation (IUGR) (Heyborne et al., 1995), highlighting the importance of distinguishing systemic effects of pregnancy from the local effect of cytokines within the fetoplacental unit.

The causes of RPL (three or more consecutive spontaneous miscarriages) are unexplained in the majority of women (Quenby and Farquharson, 1993) and it is thought that abnormalities in the immune system may have a role in idiopathic RPL (Lim et al., 1996). Increased production by PBMC of Th1-type cytokines and decreased levels of Th2-type cytokines have been demonstrated in non-pregnant women with RPL (Hill et al., 1995) and in RPL women at the time of miscarriage (Raghupathy et al., 1999, 2000).

If the shift from Th1 to Th2 cytokine response is a characteristic of normal pregnancy, a failure of this shift to occur may predispose to miscarriage in women suffering RPL. In this prospective study, the cytokine profile of PBMC from pregnant RPL women was measured and compared with the cytokine profile of pregnant women of matched gestational age with a normal obstetric history and non-pregnant women. The profile of RPL women who subsequently miscarried was also compared with those who subsequently had a live birth.

**Materials and methods**

**Patients and controls**

Local ethical committee approval was obtained for this study. Venous blood samples (10 ml) were obtained from 46 pregnant women attending the Recurrent Miscarriage Clinic at the Liverpool Women’s Hospital having previously suffered at least three consecutive miscarriages. Patients were asked to phone the clinic as soon as they realised that they were pregnant and were seen as soon as possible thereafter. They were recruited into the study if an ultrasound scan showed an amniotic sac containing a yolk sac or an intrauterine fetus between 6–10 weeks gestation. Patients were seen fortnightly with ultrasonography for reassurance until 12 weeks gestation and subsequently received routine antenatal care. Patients were excluded from the study if one of the following causes for their miscarriages was found; antiphospholipid syndrome, endocrine cause (oligomenorrhea, abnormal thyroid function tests), chromosomal cause (maternal or paternal balanced translocation) or uterine structural abnormality (assessed by cervical weakness only) (Drakeley et al., 1998). One patient was sampled at eight weeks gestation when a fetal heartbeat was detected and two weeks later when death in utero was diagnosed.

A total of 25 healthy pregnant women undergoing elective termination of pregnancy at 6–10 weeks gestation were recruited as gestationally age-matched controls. These women had no previous history of miscarriage. Eleven non-pregnant women also donated blood samples at random points in the menstrual cycle.

**Cytokine production**

A total of 10 ml blood was collected in vacutainer tubes containing preservative-free heparin (Sarstedt, Leicester, UK) and peripheral blood mononuclear cells (PBMC) isolated using Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Cells were washed twice by centrifugation in phosphate buffered saline and the number of viable PBMC determined by Trypan Blue exclusion. Cells were resuspended at a concentration of 1×10^6/ml in RPMI-1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mmol/l glutamine (Gibco Life Technologies Ltd., Paisley, UK) and 10% fetal bovine serum (Sigma, Poole, Dorset, UK). A total of 1×10^6 cells per well were cultured in 24-well plates (Nunc, Life Technologies, Paisley, UK) for 72 h at 37°C, 5% CO₂ and 85% relative humidity in the presence or absence of 5 μg/ml phytohaemagglutinin (PHA), (Murex, Biotech, Dartford, UK). The cell culture medium was then harvested and replicates pooled, aliquoted and stored at –70°C for subsequent analysis.

**Measurement of cytokines**

Cytokine production was measured in duplicate, at one or more concentrations, using enzyme linked immunoabsorbant assay (ELISA). IL-4, IL-10 and IL-12 were measured using matched pairs of capture and detection antibodies obtained from R & D

### Table I. Median cytokine production by PBMC from non-pregnant women and pregnant women with and without recurrent miscarriage. Cells were cultured in vitro and stimulated with PHA. Numbers in brackets refer to range

<table>
<thead>
<tr>
<th></th>
<th>Non-pregnant (n = 11)</th>
<th>Pregnant (n = 25)</th>
<th>RPL patients (n = 46)</th>
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<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>PHA stimulated</td>
<td>Unstimulated</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>540 (0–809)</td>
<td>5230^{+} (1850–8460)</td>
<td>719 (0–2980)</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>0.018 (0–0.3)</td>
<td>118^{+} (32.1–270)</td>
<td>0.12 (0–4.2)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>723 (17–1625)</td>
<td>1667^{+} (260–9467)</td>
<td>675 (0–2507)</td>
</tr>
</tbody>
</table>

PBMC = peripheral blood mononuclear cells; PHA = phytohaemagglutinin.

*P = 0.006 Non-pregnant versus pregnant controls.

*P = 0.003 Non-pregnant versus RPL.

*P = 0.019 Non-pregnant versus RPL.

*P = 0.026 Non-pregnant versus RPL.

*P = 0.005 Pregnant versus RPL.
Cytokines in recurrent pregnancy loss

Systems (Abingdon, Oxford, UK). TNF-α and IFN-γ were measured using commercial kits according to the manufacturer’s instructions (Genzyme, Cambridge, MA, USA) and developed using streptavidin-HRP (Zymed, San Francisco, USA) and tetramethyl benzidine (Sigma, Poole, UK). Absorbance was measured at wavelengths of 450 nm and 550 nm using a Titertek Multiskan Plus plate reader. A standard curve was constructed for each cytokine. The limit of detection of these assays was 15.6 pg/ml (TNF-α and IFN-γ), 31.25 pg/ml (IL-4 and IL-10) and 7.8 pg/ml (IL-12). Cytokine production was calculated by determining the difference between PHA-stimulated and non-stimulated cells.

Statistical analysis
Statistics were calculated using Arcus software (Cambridge, UK). The data were found to be non-normally distributed and hence the non-parametric Mann–Whitney U-test was applied to compare differences between experimental groups. Two-sided t-tests with 95% confidence intervals were calculated with a level of significance defined at $P < 0.05$.

Results
Of the 46 pregnant recurrent miscarriage patients recruited in this study, 33 subsequently produced a live birth and 13 miscarried. The production of cytokines by in-vitro stimulated PBMC was measured in blood samples obtained from pregnant women with RPL and compared with normal pregnant women of matched gestational age and non-pregnant controls (Table I). Basal levels of cytokine production by unstimulated cultured PBMCs were significantly increased upon stimulation with PHA.

Interestingly, differences were observed in cytokine production, which were subsequently reflected in pregnancy outcome (Figure 1). The level of TNF-α produced by pregnant women was significantly lower than for non-pregnant controls ($P = 0.006$), (Table I, Figure 1a). This reduction was more marked in RPL patients ($P = 0.0003$, Table I). Whereas RPL women with a successful reproductive outcome had similar concentrations of TNF-α to pregnant women, RPL women who subsequently miscarried had significantly lower levels than either pregnant ($P = 0.02$) or non-pregnant controls ($P = 0.0004$).

Production of IFN-γ was lower in pregnant than in non-pregnant women and even lower in RPL pregnant women. Statistical significance was reached when comparing RPL patients and non-pregnant controls ($P = 0.0191$, Table I). There was no difference in IFN-γ between RPL patients who subsequently miscarried and those who subsequently had a live birth (Figure 1b). Detectable levels of IL-12 were found in only three RPL patients and three controls (data not shown).

IL-10 was increased in pregnant women compared with non-pregnant controls, although this did not reach the selected criterion for significance ($P = 0.0686$). Levels of IL-10 were significantly increased in RPL patients compared with pregnant ($P = 0.005$) and non-pregnant controls ($P = 0.026$, Table I). Similar levels of IL-10 were found in RPL patients who subsequently miscarried, compared with RPL patients who subsequently had a live birth (Figure 1c).

IL-4 production was also measured in sub-sets of pregnant women ($n = 15$) and those with RPL ($n = 19$). Levels of IL-4 were significantly increased in women with RPL (median
100 pg/ml, range 20–337; \( P < 0.0001 \) compared with pregnant controls (no detectable IL-4 in any sample). Again, similar levels of IL-4 were observed in RPL patients who subsequently miscarried \( (n = 10; \text{median } 107 \text{ pg/ml, range } 20–337) \) compared to those who had a live birth \( (n = 9; \text{median } 100 \text{ pg/ml, range } 75–190) \).

One patient was sampled twice. The first sample was taken at eight weeks gestation when a fetal heartbeat was detected. At this time IFN-\( \gamma \) and TNF-\( \alpha \) levels were similar to those in pregnant controls although IL-10 was slightly increased. The patient was sampled 2 weeks later when fetal death \textit{in utero} was diagnosed. IFN-\( \gamma \) was reduced by 90\% and IL-10 was reduced by 50\%. TNF-\( \alpha \) levels were unchanged.

**Discussion**

It has been proposed that during pregnancy, systemic maternal immune responses are biased in favour of a Th2 cytokine profile (Wegmann \textit{et al.}, 1993). Our data provides evidence to support this hypothesis and confirms published data that suggests that this is also the case in humans during normal pregnancy (Marzi \textit{et al.}, 1996; Makhseed \textit{et al.}, 1999; Raghupathy \textit{et al.}, 1999, 2000). However, our hypothesis that a failure of this Th1 to Th2 cytokine shift occurs in RPL and precedes a miscarriage in some of these women was not supported; on the contrary, a Th2 profile may be exacerbated.

In this study TNF-\( \alpha \) was significantly decreased in normal first trimester samples compared with non-pregnant controls. However, in RPL women this decrease was accentuated and was even lower in RPL women who subsequently miscarried. TNF-\( \alpha \) is an important immunoregulatory cytokine, which may be produced in Th1 or Th2-type responses and is known to have different effects depending on gestational age. It has been shown that TNF-\( \alpha \) production by PBMC is suppressed at the mRNA level during early pregnancy and a significant increase does not occur until the eighth month of gestation (Tranchot-Diallo \textit{et al.}, 1997). TNF-\( \alpha \) production in late pregnancy is implicated in the induction of labour in mice (Chaout \textit{et al.}, 1990) and humans (Vince \textit{et al.}, 1992). Placental production of soluble receptors has been shown to modulate TNF-\( \alpha \) function in pregnancy (Austgulen \textit{et al.}, 1992).

IFN-\( \gamma \) production was decreased in normal first trimester samples compared with non-pregnant controls, although this did not reach statistical significance. However, in RPL women this decrease was accentuated. No differences were observed between the level of IFN-\( \gamma \) in those RPL patients who successfully completed their pregnancy compared with those who went on tomiscarry.

Cytokine production by mitogen-stimulated PBMC from women in the first trimester of pregnancy showed an increase in IL-10 compared with non-pregnant controls. Although these differences did not reach statistical significance they are in agreement with similar documented changes (Marzi \textit{et al.}, 1996) which were augmented as pregnancy progressed. A systemic reduction in IL-10 at the time of implantation has been reported in mice (Delassus \textit{et al.}, 1994). Both IL-10 and a second Th2 cytokine, IL-4, were significantly increased in women with RPL compared with pregnant controls although we did not detect any differences between the level of IL-10 or IL-4 in those RPL patients who successfully completed their pregnancy or who went on to miscarry.

Cytokines do not act in isolation but form a complex regulatory network in which modulatory interactions maintain homeostasis between the fetal unit and the maternal immune system. This complex interplay between maternal and fetal immune mechanisms also changes temporally as pregnancy progresses (Marzi \textit{et al.}, 1996; Tranchot-Diallo \textit{et al.}, 1997). If this delicate balance is adversely affected, immunoregulatory mechanisms may be insufficient to restore homeostasis and this may lead to pregnancy failure. The central role of TNF-\( \alpha \) in pregnancy may be important in determining the outcome of pregnancy in RPL women whose immunoregulatory network may be compromised before pregnancy occurs.

We found a greater Th1 to Th2 shift in pregnant women with RPL compared with pregnant controls of similar gestational age. This is in variance with other published studies (Makhseed \textit{et al.}, 1999; Raghupathy \textit{et al.}, 1999, 2000). However, these authors had taken samples after the miscarriage had been diagnosed, when the observed cytokine shift may have been the result rather than the cause of the miscarriage. The results from one RPL patient who was sampled twice, once when the pregnancy was apparently healthy according to the ultrasound and once after it had failed, add evidence to this possibility. In this patient the first sample exhibited a cytokine profile with an exacerbated Th1–Th2 shift when compared with normal pregnant women, and the second (after the fetus had died) an apparent 50–90\% change in the Th2/Th1 balance. Other authors that found a lack of cytokine shift had sampled women having a spontaneous miscarriage rather than RPL (Marzi \textit{et al.}, 1996). The pattern of cytokine production varies throughout pregnancy (Marzi \textit{et al.}, 1996; Tranchot-Diallo \textit{et al.}, 1997) and this should be taken into consideration when comparing data from other studies where the control group was in labour at term (Makhseed \textit{et al.}, 1999).

In this study we have examined the peripheral, systemic immune response in early pregnancy. A similar and equally complex cytokine balance occurs within the endometrium and decidua (Lim \textit{et al.}, 1996). The degree of interaction between the two systems is yet to be determined. Peri-implantation endometrium was found to have a predominance of Th2 cytokines (Krasnow \textit{et al.}, 1996; Lim \textit{et al.}, 1998), and in pregnancy a 10-fold increase in decidual Th2 cytokine secretion occurred (Krasnow \textit{et al.}, 1996). Women with RPL were found to have a Th1 cytokine profile in peri-implantation endometrium however, this profile did not predict pregnancy outcome (Lim \textit{et al.}, 2000). In RPL women who had miscarried, more TNF-\( \alpha \) was found in the decidua (Vives \textit{et al.}, 1999) but less in the trophoblast (Lea \textit{et al.}, 1997).

Cytokine production by T-cell clones derived from both the decidua and blood of women with RPL has been compared with that from non-pregnant women and those undergoing elective termination of pregnancy (Piccini and Romagnani, 1996). Although the majority of clones from all three patient groups showed a Th0-like profile (i.e. a pattern of cytokine production common to both Th1 and Th2 cells), a significantly higher number of Th1-type clones was generated from the decidua of women with RPL. In contrast, there were no
differences in the cytokine profile of clones generated from peripheral blood. There was also decreased production of leukaemia inhibitory factor, IL-4 and IL-10 by decidual T cells in women with RPL (Piccini et al., 1998). Maternal hormones may also be important regulators of cytokine production during pregnancy. Progesterone favours development of T cells producing Th2-type cytokines and even induces transient IL-4 production in established Th1 cells (Piccini et al., 1995). Hormone production could be responsible, at least in part, for the increased production of Th2-type cytokines implicated in maintenance of successful pregnancy.

PBMC from women with RPL produced increased levels of IFN-γ and reduced IL-10 and IL-4 when stimulated with trophoblast antigens (Hill et al., 1995). These observations were made on non-pregnant RPL patients, which indicate that an altered profile of secretion may predispose to problems in early pregnancy. We have recently described differences in endometrial immune cell populations in RPL women prior to conception which are exacerbated in those women which subsequently went on to miscarry (Quenby et al., 1999). This suggests that the immune system of RPL women may indeed be compromised before pregnancy occurs. The nature of this immune compromise is unclear. Genetic polymorphisms have been identified in genes coding for cytokine production (Wilson et al., 1992; Perrey et al., 1999; Pravica et al., 1999a,b). However, these polymorphisms have not been found to be more prevalent amongst women with RPL (Babbage et al., 2001; Reid et al., 2001).

Recurrent pregnancy loss is a pleiotropic condition with several as yet unidentified causes. An aberration in cytokine production is implicated as a contributing factor, however further study is required to characterize its role more precisely.

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