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Maternal Peripheral Blood Lymphocyte Subpopulations in Normal and Pathological Pregnancies

Key Words
Flow cytometry
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Abstract
Flow cytometry was used to determine lymphocyte subpopulations in maternal blood from 143 pathological pregnancies: 50 with fetal aneuploidy; 32 with missed abortions; 12 with ectopic pregnancies; 20 with multi-fetal pregnancies, and 29 with pregnancies complicated by intrauterine growth retardation (IUGR). The values were compared to those of 240 women with normal singleton pregnancies at 8–40 weeks of gestation and 20 non-pregnant controls. In early pregnancy (8–10 weeks), compared to non-pregnant values, there was a decrease in the percentage of CD4+ cells and CD4+ to CD8+ ratio and an increase in the percentage of CD8+ cells. In later pregnancy, the CD4+ cell percentage and CD4+ to CD8+ ratio increased and the CD8+ cell percentage decreased to reach non-pregnant values at term. The percentage of natural killer (CD3– and CD16/56+) cells decreased with gestation, while the percentage of B (CD19+) cells did not change significantly. In IUGR, the percentage of CD4+ cells and CD4+ to CD8+ ratio were decreased, while the percentage of CD8+ cells was increased. In contrast, in the groups of missed abortions and ectopic pregnancies, the CD4+ to CD8+ ratio was increased. In multifetal pregnancies and those with fetal aneuploidies there were no significant differences in maternal lymphocyte subpopulations from normals.

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Table 1. List of the monoclonal antibody panel used to enumerate maternal lymphocyte subpopulations, showing cluster designations (CD No.), alternative nomenclature and reactivity/specificity

CD No.	Alternative nomenclature	Reactivity/specificity
CD3	Leu 4, UCHT1, OKT3	T-cell receptor, Pan T-cell marker
CD19	Leu 12	Pan B-cell marker
CD4	Leu 3a	T-helper/inducer lymphocytes
CD8	Leu 2a	T-suppressor/cytotoxic lymphocytes
CD16	Leu 11	NK (CD3–) cells
CD56	Leu 19, NKH-1	NK (CD3–) cells and cytotoxic T (CD3+) lymphocytes

Introduction

Pregnancy is associated with alterations in the maternal immune system [1–4]. This study examines normal and pathological pregnancies to determine whether these alterations are reflected in altered maternal circulating lymphocyte subpopulations.

Patients and Methods

This was a cross-sectional study of peripheral venous blood from 7 groups of women. Flow cytometric analysis was performed on the same day of blood sampling. Group 1 comprised 240 women with singleton pregnancies attending routine antenatal clinics at 8–40 weeks of gestation; in all cases included in this study there were no pregnancy complications and healthy infants were born at term. In group 2 there were 20 non-pregnant, healthy female volunteers who were not using hormonal contraception or any other medication and had not been pregnant in the preceding 2 years.

Group 3 comprised 50 pregnancies at 10–36 weeks of gestation with fetal aneuploidy (trisomy 21, n = 24; trisomy 18, n = 8; trisomy 13, n = 8; Turner syndrome, n = 6, and triploidy, n = 4). The diagnosis was made by chorion villous sampling, amniocentesis or cordocentesis. The indication for karyotyping was maternal age (n = 5) or the presence of one or more of the following markers for chromosomal abnormality (n = 45): strawberry-shaped head; brachycephaly; holoprosencephaly; choroid plexus cysts; nuchal translucency; nuchal cystic hygromata; cardiac defects; diaphragmatic hernia; hydronephrosis; exomphalos; digital abnormalities, and talipes.

Group 4 comprised 32 pregnancies at 8–12 weeks of gestation, in which missed abortion was diagnosed during routine ultrasound examination. In group 5, there were 20 multi-fetal pregnancies at 8–13 weeks of gestation, including 9 triplets, 6 quadruplets, 3 pentuplets and 2 sextuplets that were referred to our centre for embryo reduction. In group 6, there were 12 tubal ectopic pregnancies at 8–9 weeks of gestation. These were diagnosed by routine early pregnancy ultrasound and were subsequently confirmed by laparoscopy.

Group 7 comprised 29 pregnancies at 21–36 weeks of gestation with intrauterine growth retarded (IUGR) fetuses due to presumed uteroplacental insufficiency. In each case the fetal abdominal circumference was below the 5th percentile for gestation and continuous wave Doppler studies (Dopptek Ltd., Chichester, UK) demonstrated an early diastolic notch in the waveform from the uterine arteries and/or absent end diastolic frequencies in the umbilical artery. None of the fetuses had any detectable malformation and the karyotype was normal.

Flow Cytometry

Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal anti-human antibodies (Becton Dickinson UK Ltd.; Oxford, UK) were used for simultaneous two-colour determination of T (CD3+), T-helper/inducer (CD4+), T-suppressor/cytotocix (CD8+), B (CD19+) and natural killer (NK; CD3– and CD16/56+) cell subpopulations (table 1).

The whole-blood method was used to stain the cells with monoclonal antibody [5]. Cytometric analysis was carried out using a FACScan and Consort 32 software (Becton Dickinson). Samples were gated using forward angle and 90° light-scattering properties to exclude granulocytes, monocytes and platelets. Gated cells were analyzed with CD14/CD45 (monocyte/leucocyte marker), to ascertain that cells were lymphoid in origin. Control staining of fetal cells with anti-mouse monoclonal IgG_{2a}-PE/IgG_1-FITC was performed on each sample, and background readings of $<1\%$ were obtained. A minimum of 5,000 cells were acquired in the lymphocyte fraction and analyzed to calculate the percentage of each subpopulation. Percentages rather than absolute numbers of lymphocyte subpopulations were used in order to avoid the effects of changes in the circulating volume during pregnancy [6].

Statistical Analysis

The data of group 1 were used to establish reference ranges with gestation. The significance of any association between the percentage of the lymphocyte subpopulations and gestational age was determined by regression analysis. Logarithmic transformation was used to make the data Gaussian. The transformed data were used to calculate the adjusted means and residual standard deviations. To determine the reference range with gestation in the original units (mean and individual 95% confidence intervals), the limits of the calculated reference range in logarithms were subjected to antilogarithmic transformation. Mann-Whitney U tests were used to determine the significance of any differences between results from non-pregnant controls and pregnant women at 8–10 weeks of gestation (n = 17) and 37–40 weeks of gestation (n = 25).

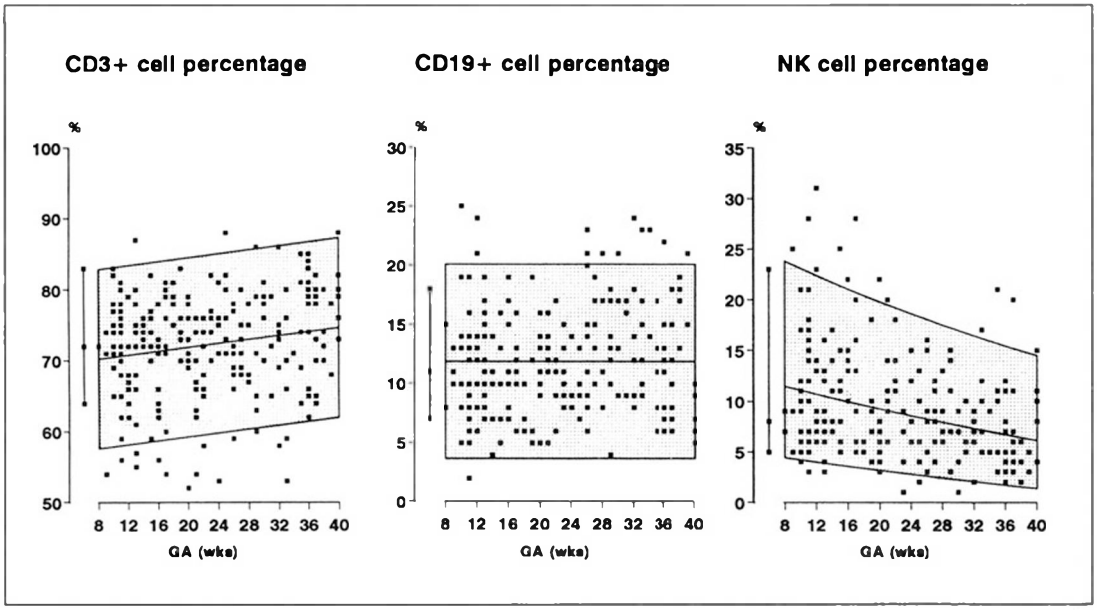
The values obtained from the groups with abnormal pregnancies were expressed as the number of standard deviations (SDs) by which the individual values differed from the appropriate normal mean for gestation (delta values). Two-tailed Student's t test was applied to determine whether the mean delta values in the maternal blood of the abnormal pregnancies were significantly different from zero.

Results

In normal pregnancy, the percentage of CD3+ and CD4+ cells and the CD4+ to CD8+ ratio increased with gestational age (fig. 1, 2; CD3+, $r = 0.171$, $p < 0.01$; CD4+, $r = 0.211$, $p < 0.01$; CD4+/CD8+, $r = 0.238$, $p < 0.001$), while the percentage of CD8+ and NK cells decreased linearly with gestation (fig. 1, 2; CD8+, $r = -0.216$, $p < 0.001$; NK, $r = -0.333$, $p < 0.0001$). The percentage of CD19+ cells did not change significantly with gestation (fig. 1; $r = 0.089$).

In early pregnancy (8–10 weeks of gestation), compared to the non-pregnant controls (group 2), there were significant differences in the CD4+ and CD8+ cell percentages and CD4+ to CD8+ ratio but not in CD3+, CD19+ and NK cell percentages (CD4+, $z = 3.35$, $p < 0.01$; CD8+, $z = -3.29$, $p < 0.01$; CD4+/CD8+, $z = 3.88$, $p < 0.001$; CD3+, $z = -0.06$; CD19+, $z = -0.47$; NK, $z = -1.77$). In late pregnancy (37–40 weeks of gestation), compared to the non-pregnant controls, there were significant differences in the CD3+ and NK cell percentages but not in the percentage of CD19+, CD4+ and CD8+ cells and CD4+ to CD8+ ratio (CD3+, $z = -2.34$, $p < 0.05$; NK, $z = 2.19$, $p < 0.05$; CD19+, $z = -0.28$; CD4+, $z = -1.57$; CD8+, $z = 1.12$; CD4+/CD8+, $z = -1.59$).

In the pathological pregnancies (groups 3–7), the significance of any differences in lymphocyte subpopulations from the appropriate normal mean for gestation (group 1) are shown in table 2. In the IUGR pregnancies, the CD4+ cell percentage and CD4+ to CD8+ ratio were significantly decreased, while the CD8+ cell percentage was significantly increased (fig. 3). In women with missed abortions there was a significant increase in the CD4+ cell percentage and CD4+ to CD8+ ratio and non-significant decrease in the CD8+ cell percentage. In women with ectopic pregnancies, there were non-significant trends similar to those observed in the group with missed abortions. In multi-fetal pregnancies and those with fetal aneuploidies there were no significant differences in maternal lymphocyte subpopulations from normals.



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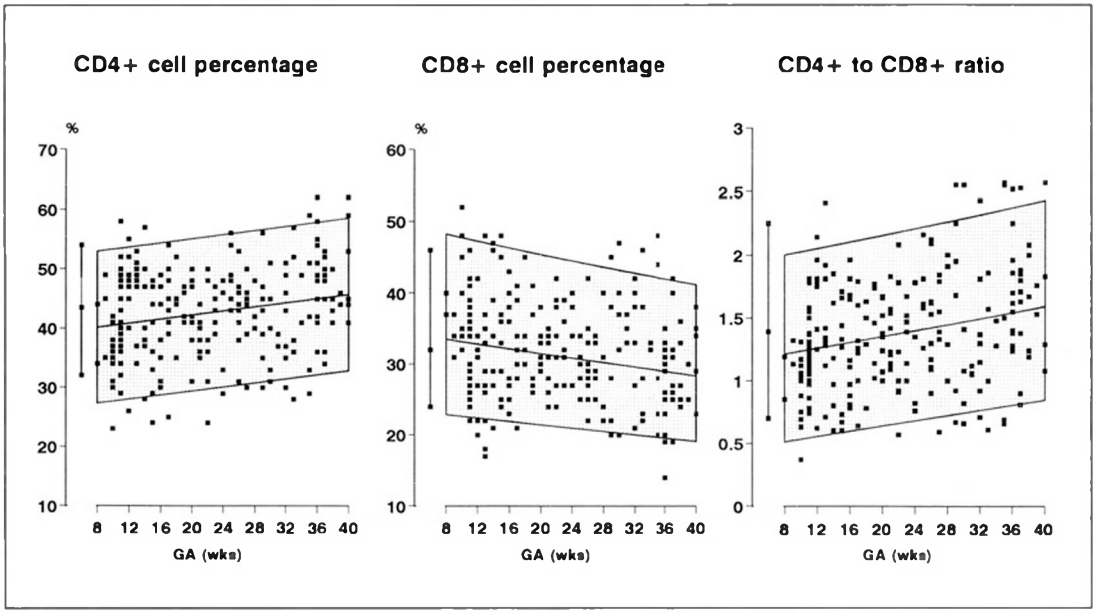

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Fig. 1. Maternal T (CD3+), B (CD19+) and NK (CD3– and CD16/56+) cell percentages plotted as a function of gestation. The sloping lines are the mean, 2.5th and 97.5th percentile values. The vertical lines represent the median and range of the non-pregnant controls.

Fig. 2. Maternal T-helper/inducer (CD4+), T-suppressor/cytotoxic (CD8+) cell percentages and CD4+ to CD8+ cell ratio plotted as a function of gestation. The sloping lines are the mean, 2.5th and 97.5th percentile values. The vertical lines represent the median and range of the non-pregnant controls.

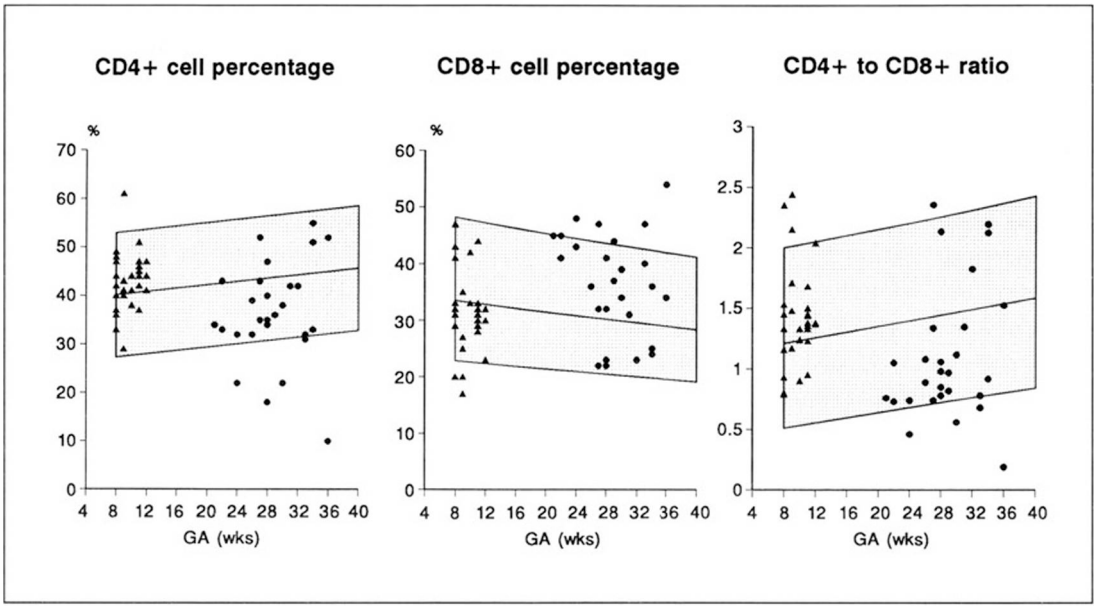

Fig. 3. Maternal T-helper/inducer (CD4+), T-suppressor/cytotoxic (CD8+) lymphocyte percentages and CD4+ to CD8+ ratio in the pregnancies complicated with missed abortion (▲) and fetal growth retardation (●) plotted on the appropriate reference range (mean, 5th and 95th percentiles) for gestation.

Table 2. The significance of any differences in lymphocyte subpopulations from the appropriate normal mean for gestation in the pregnancies complicated by fetal growth retardation (IUGR), missed abortion, fetal aneuploidy, and ectopic and multi-fetal pregnancy

	CD3+ cell	CD19+ cell	NK cell	CD4+ cell	CD8+ cell	CD4+/CD8+
IUGR	$t = -0.68$	$t = -0.38$	$t = 1.26$	$t = -3.77$ $p < 0.001$	$t = 3.40$ $p < 0.01$	$t = -3.59$ $p < 0.01$
Missed abortion	$t = 0.27$	$t = 1.55$	$t = -1.15$	$t = 2.09$ $p < 0.05$	$t = -1.60$	$t = 2.15$ $p < 0.05$
Ectopic pregnancy	$t = -0.72$	$t = 0.60$	$t = -0.76$	$t = 1.76$	$t = -1.88$	$t = 1.96$
Fetal aneuploidy	$t = -0.08$	$t = 1.12$	$t = 0.14$	$t = -0.51$	$t = -1.29$	$t = 1.09$
Multi-fetal	$t = -0.44$	$t = 1.29$	$t = -0.07$	$t = -1.06$	$t = -0.95$	$t = 0.11$

Discussion

The findings that in normal pregnancy the percentages of maternal blood CD3+ and CD4+ cells and the CD4+ to CD8+ ratio increase with gestation, while the percentages of CD8+ and NK cells decrease, demonstrate that pregnancy modulates the maternal immune system. In contrast to our findings, previous studies have either failed to demonstrate changes in lymphocyte subsets during pregnancy [7–10], or have shown a decrease in the percentage of CD3+ and CD4+ cells with gestation [11, 12]. This discrepancy may either be a consequence of the small number of patients used in previous studies [8–10, 12] or a result of the more inaccurate techniques used to enumerate lymphocyte subpopulations [7, 11].

Three previous studies have used flow cytometric techniques to assess circulating maternal lymphocyte subpopulations; Stagnaro-Green et al. [12], in a longitudinal study of 28 women during the second and third trimester of pregnancy, showed a decrease in the percentage of CD4+ cells and CD4+ to CD8+ ratio and an increase in the percentage of CD8+ cells with gestation. In contrast, Cheney et al. [9] and Iwatani et al. [13], in cross-sectional studies of 50 and 94 women, respectively, at 7–40 weeks, did not find any changes with gestation in lymphocyte subpopulations, except for an increase in the percentage of cytotoxic CD3+ cells and a decrease in the percentage of the CD4+ cells in the first trimester of pregnancy [13]. Although the latter studies showed trends similar to the findings of our study, they did not reach statistical significance probably as a consequence of analysis of the data in trimesters rather than as a function of gestational age.

The findings of a significant decrease in CD4+ cell percentage and CD4+ to CD8+ ratio at 8–10 weeks of gestation compared to the non-pregnant controls suggest that pregnancy may be associated with immunosuppression which is greatest in the first trimester. Several decidual and feto-placental products, such as placental protein-14 (PP-14), alpha-fetoprotein (AFP), progesterone, oestriol, pregnancy-associated placental protein-A (PAP-A), pregnancy-specific β1-glycoprotein (SP1), and human placental lactogen have been shown to exhibit immunomodulatory activity, and may be responsible for the maternal lymphocyte changes observed in this study [14–16]. However, our findings that the CD4+ cell percentage and CD4+ to CD8+ ratio increase with gestation to reach pre-pregnancy values at term suggests that the immunosuppressive effects of early pregnancy are reversed with advancing gestation. The only immunosuppressive pregnancy-associated hormone which peaks in early pregnancy and subsequently decreases with gestation is PP-14 [15, 17].

The finding that the percentage of maternal blood NK cells falls with gestation, parallels the changes in the number of decidual NK-like large granular lymphocytes [18, 19], and supports the hypothesis that these large granular lymphocytes are derived from maternal blood NK cells [20]. It has been suggested that these cells may play an important role in limiting the extent of trophoblastic invasion into maternal tissues, and the decrease with gestation in NK cells demonstrated in this study may be required for normal placentation and trophoblastic invasion [18, 19, 21]. The findings that fetal NK cell number and τ-interferon concentration also decrease with gestational age suggest that τ-interferon may be a common mediator for NK cell changes in the maternal, decidual and fetal compartments [22, 23].

In women with missed abortions or ectopic pregnancies, the CD4+ to CD8+ ratio is similar to that observed in non-pregnant women and is higher than in normal early pregnancy.

Since in both of these conditions there is abnormal decidualization, it could be postulated that the physiological decrease in the ratio observed in early pregnancy is a consequence of decidual factors. For example, ectopic pregnancy is known to be associated with decreased levels of PP-14 [24].

Supportive evidence for the contribution of decidual rather than feto-placental factors in the immunomodulation of early pregnancy is provided by the finding that, in multi-fetal pregnancies, there are no significant differences in maternal lymphocyte subpopulations from normal singleton pregnancies. In multifetal pregnancies, the maternal plasma concentrations of the decidual hormones are similar to those of singleton pregnancies [25]. In contrast, there is an increase in the maternal plasma concentration of several feto-placental products such as AFP, oestriol, progesterone, human placental lactogen, PAP-A and SP1.

In pregnancies with a wide variety of fetal aneuploidies there were no significant changes in maternal lymphocyte subpopulations. This finding also points to the decidua as the source of the maternal immunomodulatory factors. Although the maternal plasma concentration of human chorionic gonadotropin, AFP, oestriol, PAP-A and SP1 are altered in chromosomally abnormal pregnancies, the concentrations of decidual peptides remain normal [26, 27].

In pregnancies complicated by IUGR due to presumed impaired placental perfusion, the CD4+ to CD8+ ratio is decreased. In this condition, there is histological evidence of poor trophoblastic invasion of the decidua [28]. It has been suggested that this poor placentation may be the result of an immunologic incompatibility between parents and resultant rejection of the fetoplacental unit by the mother [29]. However, if the model of IUGR is analogous to a rejection phenomenon, one would expect an increase rather than a decrease in the CD4+ to CD8+ ratio. Furthermore, the trophoblast does not express classical MHC antigens, making immune recognition and rejection unlikely [1, 30]. If indeed there was an underlying immunological problem, it is much more likely that this would be reflected at the trophoblast-decidual interface than in maternal blood. Therefore, the observed changes are more likely to be the consequence rather than the cause of the impaired placental perfusion and IUGR. Although in this condition the PP-14 concentration has been reported to be normal, there is a strong relationship between increased levels of this hormone, hence increased immunosuppression, and low birthweight [31].

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