Leukocyte activation in the decidua of chromosomally normal and abnormal fetuses from women with recurrent abortion

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As part of our continuing programme to investigate immunological causes of unexplained recurrent pregnancy losses, we studied subpopulations of white blood cells and their activation status in decidua of women with a history of recurrent spontaneous abortion (RSA). We differentiated specifically between normal karyotyped male fetuses and abnormal karyotyped fetuses with trisomy 16 because trisomy 16 is not compatible with life and is thus a non-controversial cause of spontaneous miscarriage. Leukocytes were counted in paraffin-embedded decidua after immunohistological staining for CD45 (LCA), CD3, CD56, CD68, CD69 and CD25. Numbers of activated versus non-activated T lymphocytes, NK cells and macrophages were compared in decidua from women with: (i) unexplained RSA who had a normal male karyotype (n = 17) miscarriage; (ii) unexplained RSA who had a trisomy 16 (n = 21) miscarriage; and (iii) normal gestationally age-matched first trimester pregnancies following elective termination procedures (n = 20). Significantly more activated leukocytes were detected in the decidua of women with unexplained RSA who had a normal male karyotype compared to the other groups (P < 0.0001). In addition, numbers of cells comprising the major leukocyte subpopulation, CD56+ NK cells, appeared reduced in the decidua of women with unexplained RSA compared to decidua from women having elective terminations. Increased numbers of activated leukocytes in the decidua of women with a history of unexplained recurrent pregnancy loss who had a normal karyotyped pregnancy provide evidence that cellular immunity may be involved in unexplained recurrent pregnancy loss.

Key words: activation markers/decidua/leukocytes/recurrent spontaneous abortion/trisomy 16

Introduction

Spontaneous abortion is the most common complication of pregnancy. Recurrent spontaneous abortion (RSA), defined as three or more pregnancy losses before 20 weeks of gestation, occurs in 3 of 1000 pregnant women (Edmonds et al., 1982). The aetiology of recurrent pregnancy loss remains largely unknown, although genetic, anatomical, endocrinological and immunological abnormalities have been implicated (Hill, 1998).

Human endometrial and decidual tissues, containing a myriad of immune and inflammatory populations (Michel et al., 1990, Klentzeris et al., 1994; Hill et al., 1995; Lachapelle et al., 1996), are immunologically dynamic. In the early proliferative phase of the menstrual cycle, T-cells are the most abundant leukocyte population in the endometrium, but they decrease to 20% of all stromal leukocytes during early pregnancy (Bulmer et al., 1991; Starkey, 1991). Macrophages are also present in endometrial tissues during all phases of the menstrual cycle, but are most abundant in late secretory endometrium and in decidual tissues during early pregnancy (Bulmer, 1996). The most abundant population (70%) of stromal leukocytes observed in late secretory endometrium and in the decidua during early normal pregnancy are large granular lymphocytes (LGL) bearing the unique phenotype: CD56+, CD16−, CD57−, CD2±, CD38±, CD3−, CD4−, CD8− (Bulmer and Sunderland, 1984; Ritson and Bulmer, 1987, 1989; Starkey et al., 1988; Bulmer et al., 1991). Increased mean numbers of CD56+ cells have been reported in secretory endometrium of women with recurrent miscarriages (Clifford et al., 1999; Quenby et al., 1999).

The hypothesis that the balance between T-cell subsets (i.e. CD4+ and CD8+ cells) plays a role in maintaining successful pregnancy is controversial. Some studies have reported no difference between the proportion of T-cell subsets in either secretory endometrium or first trimester decidua from women with a history of recurrent pregnancy loss compared with
normal controls; while others have described a shift towards a higher ratio of CD8+ T-cells in endometrial biopsies from women with a history of recurrent pregnancy loss (Klentzeris et al., 1994; Hill et al., 1995; Lachapelle et al., 1996; Quenby et al., 1999).

The activation status of T-cell populations may provide another indicator of cellular immunity potentially involved in pregnancy loss. One such marker, the interleukin (IL)-2 receptor is a universal T-cell activation marker, consisting of three chains, IL-2Rα (CD25), IL-2Rβ and IL-2Rγ (Smith et al., 1988; Ishii et al., 1994). These three chains together form a high affinity receptor for IL-2, a cytokine which stimulates T-cell proliferation. The IL-2 receptor has been reported to be absent on T-cells in normal first trimester decidual tissues using immunohistochemical techniques (Bulmer and Johnson, 1986); however, using flow cytometry, CD3+CD25+ activated T-cells have been identified in decidual tissue (Saito et al., 1992). CD69 is another early activation marker expressed on the surface of T-cells (Testi et al., 1989). A proportion of T-cells constitutively expresses this marker throughout the menstrual cycle and in normal early pregnancy. Flow cytometry studies indicate that in first trimester decidua, a proportion of T-cells (58% CD4+ cells and 73% CD8+ cells) express CD69 (Maruyama et al., 1992; Saito et al., 1992; Vassiliadou et al., 1998).

Approximately 60% of spontaneous abortions are attributable to numerically abnormal chromosomes in the developing conceptus (Boue et al., 1975), yet no study to date addressing immunological phenomena in the decidual of women with RSA has compared their data relative to well-defined chromosomal abnormalities belonging to conceptuses. This is important since the chromosome abnormality trisomy 16, which occurs in 26% of all spontaneous abortions, is incompatible with life (Benn, 1998) and would thus be an undisputed cause of spontaneous abortion. Therefore, the purpose of our study was to determine whether decidual leukocyte subpopulations and their associated activation markers were different between women having recurrent pregnancy loss of either a trisomy 16 conceptus (47XY+16 or 47XX+16) compared with a chromosomally normal male conceptus (46XY) and compared with gestationally age-matched decidual tissues from women having elective pregnancy termination. Samples from presumably normal female conceptuses (46XX) were not chosen for study because of the inability to accurately differentiate from possible maternal cell contamination.

Materials and methods

Tissues
For this study, we chose decidual tissues that were obtained immediately from 58 women having a dilation and evacuation procedure (D&E) between 6 and 10 weeks of gestation at Brigham and Women’s Hospital. Of these 58 women, 38 had a D&E for a repeat missed abortion (MAB). All of these 38 women had a history of at least three prior first trimester miscarriages of unexplained aetiology (normal parental karyotypes, intrauterine structural study, luteal phase endometrial biopsy, hormone concentrations and negative cervical cultures, lupus anticoagulant and antibodies to cardiolipin and phosphatidyl serine). The fetal karyotypes were trisomy 16 in 21 and 46XY in 17 of the 38 women chosen with a MAB. An additional 20 women who had elective pregnancy termination were gestationally age-matched with the recurrent pregnancy loss group as a further control. In all 58 cases, fetal heart activity had been identified within 2 weeks of tissue collection. Decidual tissues were fixed in 10% unbuffered methanol-free formalin, embedded in paraffin and cut into 5 µm sections.

Monoclonal antibodies
The seven mouse monoclonal antibodies (mAb) used to identify leukocyte types in paraffin sections are summarized in Table I.

Immunohistochemistry
Serial sections (5 µm) were deparaffinized in ProPar® (Anatech, Ann Arbor, MI, USA) and rehydrated through descending grades of alcohol and several changes of distilled water. All sections were subjected to an antigen unmasking procedure that was carried out by heating the slides in a target retrieval solution, pH6. (Dako, USA) for 20–30 min at 99°C followed by cooling for 20 min, except for CD45 and CD68, which were pretreated with the digestive enzyme trypsin (1.25 mg/ml; Biogenex, San Ramon, CA, USA) for 20 min at 37°C.

Blocking of endogenous peroxidase was performed using 0.05% hydrogen peroxide (Fisher, Fair Lawn, NJ, USA) in methanol for 10 min. Endogenous biotin was minimized using the Biotin Blocking System (Dako) according to the manufacturer’s instructions. For CD25 the blocking times were doubled and an extra blocking step with 0.1 mol/l sodium azide (Sigma, St Louis, MO, USA) in phosphate buffered saline (PBS) for 20 min was included.

For all monoclonal antibodies except CD25, the Vectastain Universal Elite® peroxidase kit (Vector Laboratories, Burlingame, CA, USA) was used. Sections were incubated with blocking serum (20 min) followed by the addition of primary antibody (Table I), secondary antibody, and biotinylated horse, anti-mouse immunoglobulin according to kit instructions. For CD25 the Catalyzed Signal Amplification Peroxidase System® (Dako) was used. Following incubation with the primary antibody, sections were incubated with biotinylated–avidin system, streptavidin–biotin complex, amplification reagent and streptavidin–peroxidase, all for 15 min at room temperature. Between reagents the sections were washed in 0.05 mol/l Tris–HCl. All mAb were visualized using 3,3’-diaminobenzidine tetrahydrochloride, containing hydrogen peroxide which stained positive cells reddish brown. The incubation time for the substrate varied between 2 and 10 min. Sections were counterstained with haematoxylin (Zymed, CA, USA), dehydrated in alcohol and mounted with non-aqueous permanent mounting.

Negative controls were performed at all levels of immunostaining and included omission of the primary mAb, secondary antibody, avidin–biotin complex, amplification reagent, streptavidin–peroxidase, which were all replaced with blocking serum. Formalin-fixed, paraffin-embedded tonsil tissues were used as positive controls.

Quantification and analysis of results
Positive cells with distinguishable nuclei were counted. Quantification was performed at ×20 magnification using a 10×10 mm reticle. A minimum of 250 CD45+ cells per slide were counted to provide an adequate number of cells for comparison using one to five medium-power (×10 eyepiece, ×20 objective) fields per section, followed by counting each slide and each mAb in the same area in serial sections.

For evaluation of differences between groups, data for CD3/CD45, CD56/CD45 and CD25/CD45 were analysed using analysis of variance (ANOVA) because these data fulfilled assumptions for parametric testing. When a significant F ratio was defined by ANOVA,
groups were further compared using Fisher protected least significant differences post-hoc testing with StatView software. All other data were not parametric. Therefore, the Kruskal–Wallis test was used. Statistical significance was assumed with $P < 0.05$.

## Results

The mean (± SD) age for the three groups was 28.7 ± 5.7 years for women having elective pregnancy terminations; 35.2 ± 5.0 years for women with recurrent pregnancy loss of a fetus with a normal male karyotype; and 36.3 ± 4.9 years for women with recurrent pregnancy loss of a fetus with trisomy 16. The estimated gestational age of pregnancy in all study groups was 8 ± 2 weeks gestation.

**CD45+ cells**

CD45+ cells (all white blood cells; WBC) were detected singly or as cellular aggregates in the stroma, and around arterioles and glands. The distribution pattern of these cells was similar in all three groups (Figure 1). In three cases from the recurrent pregnancy loss group with a normal karyotype an increased number of accumulated cells arranged in follicles were identified, but the number of CD45+ cells detected per high power field was not significantly different between groups ($P = 0.32$). The non-parametric distribution of cells was 121.5 (median; range 40.3–304) positive cells in normal first trimester decidua, 89.7 (median; range 46.8–202) in decidual samples from women with recurrent pregnancy loss having loss due to trisomy 16 and 102.3 (median; range 57.7–283) positive cells for samples from women with recurrent pregnancy loss who had a normal fetal karyotype (Figure 2).

**CD56+ cells**

Large granulated lymphocytes formed the largest group of stromal leukocytes identified and were similarly distributed uniformly through the tissue with no focal accumulations detected (Figure 1). Concentrations of these cells appeared higher in the control group, although the differences were not statistically significant. The control group contained a median of 62.5 (range 22.4–189) positive cells per high power field compared with a median of 44 (range 19.2–99) in recurrent aborters with an abnormal karyotype and 44.5 (range 7–205) in women with recurrent pregnancy loss of a chromosomally normal fetus ($P = 0.08$; Figure 2). A significant difference ($P < 0.05$) was detected in the ratio of CD56+ to CD45+ ratio in the decidua from women having elective pregnancy terminations was 56.6 ± 2.6% while in women with recurrent pregnancy loss of a chromosomally normal conceptus it was 43.8 ± 4.3%. There was no statistically significant difference between the CD56+ to CD45+ ratio in women having recurrent pregnancy loss with an abnormal karyotype (48.9 ± 3.6%) compared with the other two groups.

**CD3+ cells**

The second most abundant population of stromal leukocytes identified in the three groups was CD3+ cells (Figure 1). A non-parametric distribution of CD3+ cells was observed in all samples. In women having elective pregnancy terminations the median number of CD3+ cells per high power field was 51.4 (range 0–131) compared to 42.3 (range 19.2–99) in recurrent aborters with an abnormal karyotype and 57 (range 21.3–124) in recurrent aborters having a chromosomally normal conceptus. These differences were not statistically significant (Figure 2). Similarly, the ratio of CD3+ cells to CD45+ stromal leukocytes was not significantly different between groups (Figure 3). The mean ratio (± SEM) for elective terminations was 43.7% ± 3.8%; for recurrent aborters with an abnormal karyotype, 49.5% ± 4.1%; and for recurrent aborters with a normal karyotype, 53.2% ± 5.4%.

**CD68+ cells**

Concentrations of macrophages were not statistically different between groups (Figure 1). Macrophages were primarily distributed as single cells in the decidua of all three groups with sporadic focal accumulations. Decidual samples from elective terminations contained 17.8 (median, range 0–66) positive cells per high power field, compared with 13.3 (median; range 2.3–37.5) in samples from recurrent aborters with an abnormal karyotype and 13.3 (median; range 0–34.5) in recurrent aborters with a normal karyotype (Figure 2).

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### Table 1. Primary monoclonal antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity/ antigen</th>
<th>Cell type identified</th>
<th>Source</th>
<th>Dilution</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dako-LCA</td>
<td>CD45</td>
<td>All WBC CA, USA</td>
<td>Dako, Carpinteria</td>
<td>1:100</td>
<td>60 min</td>
</tr>
<tr>
<td>NCL-CD3-PSI</td>
<td>CD3</td>
<td>T-cells</td>
<td>Novocastra, Burlingame, CA, USA</td>
<td>1:75</td>
<td>60 min</td>
</tr>
<tr>
<td>Anti-Leu-19</td>
<td>CD56</td>
<td>NK cell LGL cells</td>
<td>Zymed, San Francisco, CA, USA</td>
<td>1:200</td>
<td>120 min</td>
</tr>
<tr>
<td>Macrosialin</td>
<td>CD68</td>
<td>Macrophages</td>
<td>Dako, Carpinteria CA, USA</td>
<td>1:80</td>
<td>60 min</td>
</tr>
<tr>
<td>FAL, Lewis X</td>
<td>CD15</td>
<td>Granulocytes</td>
<td>Dako, Carpinteria CA, USA</td>
<td>1:100</td>
<td>120 min</td>
</tr>
<tr>
<td>Leu-23</td>
<td>CD69</td>
<td>Activated T-cells</td>
<td>Serotec, Raleigh, NC, USA</td>
<td>1:50</td>
<td>Overnight</td>
</tr>
<tr>
<td>IL2α receptor</td>
<td>CD25</td>
<td>Activated T-cells</td>
<td>Dako, Carpinteria CA, USA</td>
<td>1:2000</td>
<td>15 min</td>
</tr>
</tbody>
</table>
Similarly significant differences were not detected between groups with respect to the ratio of CD68+ to CD45+ cells in the decidua. The ratio of CD68+ to CD45+ cells was 17.5% (mean; 2.9% SEM) in the control group versus 12.7% (mean; 2.4% SEM) in women with recurrent pregnancy loss having an abnormal karyotype in the conceptus and 14.1% (mean; 2.9% SEM) in recurrent aborters with a normal fetal karyotype.

**CD25+ cells**

The numbers of IL-2α receptor positive cells (CD25+) were very low per high power field and to confirm positive findings, entire sections were counted (Figure 1). The number of IL-2α receptor (CD25) positive cells, although low (2.0 median, range 0.5–5) in decidual samples of women with recurrent pregnancy loss having a chromosomal normal conceptus, was nevertheless significantly higher ($P < 0.0001$) than in the decidua from either women having elective pregnancy terminations (0.03 median, range 0–3) or women with recurrent pregnancy loss having a trisomy 16 conceptus (0.2 median, range 0–5) (Figure 2).

The ratio of CD25+ cells to CD45+ leukocytes also revealed a significant difference ($P < 0.0001$) between groups. The ratio of IL-2α receptor bearing cells to all white blood cell populations from elective pregnancy terminations was $0.3 \pm 0.1\%$ (±SEM) and for recurrent aborters with an abnormal karyotype $0.5 \pm 0.2\%$ (±SEM), whereas in the group of recurrent aborters with a normal karyotype the ratio was $2.0 \pm 0.3\%$ (±SEM).

**CD69+ cells**

No significant differences were observed in either the numbers or the proportions of CD69+ cells between groups (Figure 1). In elective terminations the median number of CD69+ cells was 11.3 (range 0.6–78); while the mean ratios of CD69+ cells to CD45+ cells was 10.0% (3.5% SEM) compared with 8.8 (median; range, 0–47) CD25+ cells and CD69+ to CD45+ ratio of 19.4% (mean; 3.6% SEM) in women with recurrent pregnancy loss with an abnormal karyotype and 17.8 (median; range 2–56.5) CD69+ cells and CD69+ to CD45+ ratio of 19.4% (mean; 6.5% SEM) in women with recurrent pregnancy loss having a normal abortal karyotype (Figure 2).
Figure 2. Numbers of positive cells per field in decidua. Group A: RSA with normal karyotype embryo, \( n = 17 \); group B: RSA with abnormal karyotype embryo, \( n = 21 \); group C: elective abortions, \( n = 20 \). There were significantly more CD25+ cells in decidua from group A than in group B and C (\( P < 0.05 \)). Box plots: horizontal lines of boxes delineate the 25th, 50th and 75th percentile and the whiskers depict the 10th and 90th percentile of the population, the notch defines the 95% confidence interval around the median; thus groups that display non-overlapping notches are significantly different (\( P < 0.05 \)).

CD15+ cells

To exclude acute inflammation all sections were stained for CD15, a granulocyte marker. Only a few granulocytes were identified (controls: median 0.0, range 0–0.05; recurrent aborters with an abnormal karyotype: median 0.0, range 0–0.2). None of the samples had evidence of acute inflammation. Neither age nor parity affected our results (data not shown).

Discussion

Immunological mechanisms have been implicated in recurrent pregnancy loss, although very little direct pathological evidence exists in humans (Hill, 1998, review). One problem with analysing tissues from miscarriages is that cause versus effect phenomena are difficult to distinguish. Therefore, it is important to compare data relative to chromosomal abnormalities belonging to conceptuses, as was carried out in this study, since a chromosome abnormality such as trisomy 16 is an undisputed cause of miscarriages. Our findings suggest a higher activation status of decidual leukocytes in women experiencing unexplained recurrent pregnancy loss with chromosomally normal (46XY) aborti compared with women with either a history of unexplained recurrent pregnancy loss with a conceptus that was chromosomally abnormal (trisomy 16) or with women undergoing gestationally age-matched elective pregnancy terminations.

In normal first trimester decidua using immunohistochemical techniques IL-2Rα (CD25) is not expressed on either CD56+ or CD3+ cells (Bulmer and Johnson, 1986; Starkey, 1991). However, using flow cytometry, CD3+CD25+ cells have been identified in normal first trimester decidua (Saito et al., 1992). Significantly higher numbers of CD25+ T-cells have been observed in the decidua of sporadic spontaneous abortions compared to elective terminations; however, karyotype analysis of abortus tissues was not performed in this prior study (Vassiliadou et al., 1999). CD56 bright cells express IL-2Rα following in-vitro stimulation (Parhar et al., 1989; King and Loke, 1990; Nishikawa et al., 1991).

Our study provides evidence that differentiation between normal and abnormal karyotyped embryos in RSA is important in investigative studies addressing mechanisms of loss. The activation status of leukocyte subpopulations as measured by...
CD25 revealed a significant difference in decidual samples depending upon whether the pregnancy was chromosomally normal or abnormal. The small, but significantly higher proportion of CD25+ cells in RSA with a normal karyotyped embryo, observed in our study, may influence pregnancy maintenance.

CD69+ cells were not significantly different between any of our study groups, although there was a tendency towards increased numbers in women with recurrent pregnancy loss having a normal karyotyped embryo. Similarly a tendency for higher numbers of T-cells in RSA with a normal karyotyped embryo compared to the other two groups was observed. A much larger investigation would be needed to address this issue.

A significantly lower ratio of CD56+ to CD45+ cells in decidua of RSA associated with normal karyotype compared to tissues obtained following elective abortion was detected, perhaps indicating a supportive role for CD56+ cells in early human pregnancy. The lower number could be due to a lack of extracellular matrix components (ligands) like laminin, fibronectin and collagen (Springer, 1990). On the other hand absence or defective expression of these ligands by endometrial tissue in RSA may result in diminished binding of CD56 cells to the endometrium. A lower percentage of decidua CD56+ cells has been reported previously in normal karyotyped miscarriages compared with abnormal karyotyped miscarriages and electively terminated pregnancies (Yamamoto et al., 1999). However, in that study it was unclear what was meant by a normal karyotype (i.e. where 46XX and 46XY miscarriages are compared). This is potentially important since a 46XX karyotype may reflect maternal cell contamination of the miscarriage sample.

The total number of LGL, T-cells and macrophages exceeded that of CD45+ cells in all sample groups which have been previously reported (Marshall and Jones, 1988; Bulmer et al., 1991). This could be due to the presence of a subgroup of CD68+ cells which do not express CD45. Unlike previous reports (Starkey et al., 1988; Bulmer et al., 1991; King et al., 1991; Vassiliadou and Bulmer, 1998), in this study, macrophages formed the third largest group of stromal leukocytes. This discrepancy may be due to pretreatment methods and different clones of antibodies used in our study; although another recent study (Marx et al., 1999) revealed the same distribution of these cells as reported by us.

In conclusion, further phenotypical studies in pathological human pregnancy and karyotype assessment of abortus material are important in defining potential causes of pregnancy loss which will facilitate assessment of immunological phenomena observed in decidual tissue so that accurate interpretations may be made.

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Leukocyte activation in the decidua according to fetal chromosomes


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