Circulating Lymphocyte Immunophenotypation by Flow Cytometry as Fast and Efficient Method for Immune Status Assessment in Second Trimester of Normal Pregnancy and Pregnancy Complicated by Miscarriage

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Abstract: The immunophenotypation of circulating lymphocyte by flow cytometry is a fast and efficient modern quantitative method and has been also used to assess immune status in normal pregnancy and pregnancy complicated by infection or with placental abnormalities or with preeclampsia. Since the literature indicates controversy in the immune etiology of abortion and in the levels of circulating lymphocyte subsets in normal pregnancy, this study aims to extend current knowledge by quantification with flow cytometry sets and subsets of circulating lymphocytes in the second trimester of normal pregnancy and pregnancy complicated by miscarriage.

Key Words: Circulating lymphocyte, Immunophenotypation, Flow cytometry, Second trimester pregnancy, Pregnancy complicated by miscarriage, Recurrent abortion

1 Introduction

The influence of pregnancy on immune function is poorly understood [1,2,3]. Although most women do not present immunodeficiency during the pregnancy the frequency and the severity of some infections suggest that some immune responses are modified[4].

So, in normal pregnancy, in contrast with the pregnancy complicated by infection with human immunodeficiency virus type1 (HIV-1), Burns and his coworkers [4] found that the lymphocytes T helper (CD4+), which are low in the first part of gestation, are increasing progressively from the third trimester, while the CD8+ cells (suppressor / cytolytic lymphocytes T) are increasing in the moment of birth.

Natural cells killer (NK) are also changing insignificantly during pregnancy compared to negative population [5,6].

In addition to the immune changes of the uterus [7,8,9], the immunology of pregnancy is difficult to interpret at the peripheral blood level. There are great individual variations of normal limits in terms of maternal circulating lymphocyte subsets [10] and the insufficient knowledge of the role of mediators of cytokines in cellular events related with the installation and maintenance of pregnancy [11,12,13,14,15,16,17]. It is difficult to interpret the immunology of pregnancy because are well demonstrated nowadays early feto-maternal transfusion [18,19], the activation of the fetal immune system in utero [20,21] and the influence of the maternal immune response to genital infection with Ureaplasma urealyticum that is responsible both for recurrent miscarriage and the polyclonal B lymphocyte activation by increasing titres of different antibodies, like the antiphospholipid [22,23]. The immunophenotypation of circulating lymphocyte by flow cytometry is a fast and efficient modern quantitative method and has been also used to assess immune status in normal pregnancy [4,10] and pregnancy complicated by infection [4] or with placental abnormalities [24] or with preeclampsia [25,26].

The same method has been exploited in the study of miscarriage [27,28,29,30,31] in conditions where independent immune disturbances and infection-induced are frequently incriminated in the etiology of abortion, especially the recurrent one [22,32,33,34,35,36,37,38,39,40,41,42], which has repercussions on therapeutic efficiency of recurrent immune abortion [38,43,44,45,46,47,48,49,50].

Because the literature indicates controversy in the immune etiology of abortion and in the levels of
circulating lymphocyte subsets in normal pregnancy, this study aims to extend current knowledge by quantification with flow cytometry sets and subsets of circulating lymphocytes in the second trimester of normal pregnancy and pregnancy complicated by miscarriage.

2 Material and Methods
This prospective and controlled study has selected 47 pregnant women with gestational age (transabdominal ultrasound confirmed) between 13-27 weeks. They were evaluated either ambulatory or interned in departments of obstetrics gynecology clinic from University of Craiova for normal pregnancy or complicated pregnancy with threat of miscarriage (diagnosis established by uterine bleeding +/- painful uterine contractions + clinical and paraclinical exclusion of placenta praevia, gestational trophoblastic disease, ectopic pregnancy and uterine and fetal malformations [34]).

After the obtaining the consent of the 47 pregnant women, the study subjects were classified in the following categories:

(a) 12 pregnant women with normal pregnancy between 13-26 weeks of gestation, identified during prenatal consultation. Their results of lymphocytic Immunophenotypation were used as control for the group of pregnant women with complicated pregnancy. On the other hand, the results were stratified analyzed (< 20 weeks of gestation or >20 weeks of gestation) to exclude the possible influences of gestational age on serum levels of lymphocyte population during the second trimester of pregnancy;

(b) 35 pregnant women in second trimester of pregnancy with threat of miscarriage that had not evolved to the expel of the product concept in the first 48 hours from the debut of uterine bleeding. They were also divided in a group with gestational age <20 weeks and another with gestational age >20 weeks.

Each of the 2 subgroups included, respectively, 8 and 5 pregnant women with history of recurrent abortions, diagnosis based on the presence of at least 3 consecutive spontaneous abortions.

The pregnant women (n=47) admitted in our study for lymphocytic Immunophenotypation were clinical and paraclinical evaluated according to current protocols [51,34,52] of which were missing in the case of pregnant women with recurrent abortion the determination of antiphospholipid antibodies, assessment diluted viper venom time Russell, cervicale uterine cultures for Ureaplasma urealyticum and Chlamidia trachomatis and the thrombophilia screening (the determination of nithrombin III, protein C, S) near the genetic test for detecting the Leiden mutation of V factor.

The exclusion criteria of our study consisted of:
(a) lupus erythematosus manifested; (b) general or local infection apparent on evaluation or most recent; (c) izoimunisation Rh; (d) serum hemolysison sample of peripheral blood taken for Immunophenotypation; (e) immunomodulatory therapy influence on sampling for flow cytometry; (f) refusal of a complex evaluation before enrollment in the study of peripheral lymphocyte sets; (g) other causes of uterine bleeding than the threat of miscarriage; (h) other causes of recurrent abortion (genetic, anatomical, fetal, endocrine, microbiological- others that Ureaplasma urealyticum and Chlamidia) and multiple pregnancy [37,39,53,54,55].

Flow cytometry is a modern method, inexpensive, relatively simple technique, through it can be determined simultaneously more physical characteristics (and morphology, the existence of specific structures recognized by the fluorochrome) of one cell moving in a liquid in conditions where cell analysis rate is between 500 and 4000 cells / second).

A value of p<0,05 was statistically significant at the comparing the results of the investigation by Student’s and Mann whitney U tests, according to the argumentative principles of Welch & Gable [56].

3 Results and Discussions
Demographic analysis rendered in Table I shows, in addition to the number of subjects in the control group and test group and the comparability of the lots and sub-lots of pregnant investigated about the average age and gestational age, while the apparent incompatibilities related to parity and gestation number may not affect the results of this immune research because it is widely agreed that after conception expel the peripheral imunocyte levels return to preconceptional status [4,10]. The comparison between sub-lots of healthy pregnant with gestational age until 20 weeks and over, about the evolution in the major subsets of peripheral lymphocytes during the second trimester of normal pregnancy, reveals no statistically significant differences.

The percentages of circulating lymphocyte subpopulations in pregnant women with threatening abortion does not change strongly after 20 weeks of gestation over the period up to 20 weeks, in the second trimester of pregnancy.
Table 1  The demography of pregnant groups investigated by flow cytometry

<table>
<thead>
<tr>
<th></th>
<th>Gestational age</th>
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<tbody>
<tr>
<td></td>
<td>13-20 weeks</td>
<td>21-27 weeks</td>
<td></td>
</tr>
<tr>
<td><strong>Number of subjects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (normal pregnancy)</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Test group (with/without history of recurrent miscarriage)</td>
<td>20 (12/8)</td>
<td>15 (10/5)</td>
<td></td>
</tr>
<tr>
<td><strong>Maternal age (average +/-standard error of average)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>23 + - 1,4</td>
<td>22.6 + - 1,76</td>
<td></td>
</tr>
<tr>
<td>Test group</td>
<td>23,7 - 0,9</td>
<td>23 + - 0,95</td>
<td></td>
</tr>
<tr>
<td><strong>Pregnancy ( number of subjects)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Primigravida</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Multigravida</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Test group</td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Primigravida</td>
<td>13 (11,75-14)</td>
<td>15 (14,25-16,75)</td>
<td></td>
</tr>
<tr>
<td>Multigravida</td>
<td>16 (14,25-44,75)</td>
<td>13 (36,25-44,75)</td>
<td></td>
</tr>
<tr>
<td><strong>Parity ( number of subjects)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Test group</td>
<td>16</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Gestational age ( average +/- standart error of average)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>17,66 + - 0,57</td>
<td>23,6 + - 1,66</td>
<td></td>
</tr>
<tr>
<td>Test group</td>
<td>16,75 + - 0,59</td>
<td>24,2 + - 0,47</td>
<td></td>
</tr>
</tbody>
</table>

Table 2  A comparison between the percentages of lymphocyte subpopulations in pregnant women with normal pregnancy evolving without history of recurrent miscarriages and those of pregnant women with a history of recurrent miscarriages

<table>
<thead>
<tr>
<th>Lymphocytic population</th>
<th>Percent (percentili median with 25 and 27)</th>
<th>Statistically significance (*Mann whitney U test and ** Student’s t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without recurrent abortions</td>
<td>With recurrent abortions</td>
</tr>
<tr>
<td>Total T lymphocytes</td>
<td>66 (65-72,5)</td>
<td>64 (55-69)</td>
</tr>
<tr>
<td>Total B lymphocytes</td>
<td>19 (14,25-22)</td>
<td>13 (10,75-16,75)</td>
</tr>
<tr>
<td>T-helper lymphocytes</td>
<td>43 (37,75-46,5)</td>
<td>44 (33,5-49,75)</td>
</tr>
<tr>
<td>Cytholytic T lymphocytes</td>
<td>32 (27,25-40,5)</td>
<td>31 (36,25-44,75)</td>
</tr>
<tr>
<td>T-helper/ T cytholytic</td>
<td>1,253 + - 0,235</td>
<td>1,230 + - 0,291</td>
</tr>
<tr>
<td>Activated B lymphocytes</td>
<td>13 (11,75-14)</td>
<td>15 (14,25-16,75)</td>
</tr>
<tr>
<td>Activated T lymphocytes</td>
<td>10 (8-10)</td>
<td>4 (2-10)</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>18 (16,25-20)</td>
<td>9 (6,5-19,75)</td>
</tr>
</tbody>
</table>
No notable differences occur between subsets of peripheral immunocyte in normal pregnancy reported to the complicated pregnancy with the threat of abortion in second trimester of pregnancy up to 20 weeks and after 20 weeks.

When one views in parallel the percentages of circulating lymphocyte subpopulations in the second trimester of, respectively, normal pregnancy and pregnancy complicated by threat of miscarriage, regardless of the gestational age, what is opposites all pregnant women with painful uterine contractions (11 from 35 abortion threats) of the test group to those of the control group (painless contractile), the threat of abortion in second trimester of pregnancy is characterized by a significant increase of CD4 + (T helper) cells and by a decrease in statistical evidence of total B lymphocytes compared to normal pregnancy in second trimester of pregnancy.

The comparison, rendered in Table 2, of the entire sub-lot of recurrent miscarriages under threat of abortion regardless of gestational age in terms of percentage levels of the main peripheral blood lymphocyte subpopulation, indicate a significant growth of the B lymphocytes activated only among pregnant women with recurrent miscarriages compared to those with uncomplicated pregnancy by abortion in the second trimester of pregnancy.

The results show that the fluctuation levels and peripheral lymphocyte subpopulation percentages during the normal pregnancy in second trimester of pregnancy are consistent with most previous studies [4,5,10] by flow cytometry in normal pregnancy. The fact that no CD4 + cell rise beginning at the end of second trimester of pregnancy, or CD8 + population growth are associated to fetal expulsion [4] are not apparent in normal pregnant women studied by us is explained by the maximum limit in the control group of 26 weeks gestational age and by the fact that no normal pregnant and with threat of abortion not expelled the fetus in the first hours after sampling the reading profile of peripheral cellular immune.

Significantly increasing of the percentage of T-helper lymphocytes near by the reducing the level of total peripheral B lymphocytes in the threat of abortion in second trimester of pregnancy, noticed by us, is not a simple redistribution of these sets of immunoocyte [57], which was detected from normal reporting of the groups with fewer pregnant women with threatening of late miscarriage. Is rather the immunomodulating effect associated to the release of prostaglandin E2 during painful uterine contractions that accompany the hipercontizolemy [58].

The only difference between the normal pregnant women of same gestational age from group with pregnancy with threat of miscarriage and a history of recurrent abortions was the significant increasing of circulating activated B lymphocyte subset. That means that by one measure, using the Immunophenotypation of peripheral lymphocytes we can make the screening of the group of recurrent abortion generate by the presence of the antiphospholipid antibodies with or without genital infection with Ureaplasma urealyticum [22] which separates it from the rest of recurrent genetic abortions associated with other defects thrombophilia [49].

Reporting our results to those from the literature [4] one could show that fenotipation of peripheral blood by flow cytometry in dynamic of pregnant women in the second trimester of pregnancy can serve as screening simple, inexpensive, practical to detect HIV -1 infection associated with pregnancy. On the other hand, specific therapy in case of infection with Ureaplasma urealyticum associated to the pregnancy, the fenotipation of the peripheral lymphocytes by flow cytometry in dynamic could be a way to monitorize the therapy efficiency.

By comparing our results obtained by flow cytometry for shaping the peripheral immune profile of pregnancy complicated with recurrent miscarriage and abortion threat in the second trimester of pregnancy with the observations on serum C-reactive protein (CRP) in the second trimester of pregnancy complicated with the same things [59] we can say that the most common mechanism of recurrent miscarriage in the study population is expressed through another variant of the cytokine cascade than the one that trains the interleukin – 6 (the main inductor of serum CRP).

### 4 Conclusion

Our study highlights that the fenotipation by flow cytometry of the peripheral lymphocytes can be a practical screening, at least for recurrent abortion of immune cause in the second trimester of pregnancy.

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