

T-HELPER 1 CYTOKINES INCREASE DURING EARLY PREGNANCY IN WOMEN WITH A HISTORY OF RECURRENT SPONTANEOUS ABORTION

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ABSTRACT

The measurement of various cytokines may provide a different tool for evaluation of the immune system. Recent investigations have shown that the number and function of peripheral natural killer cells (NK- cells) increase during early pregnancy in women with a history of unexplained recurrent spontaneous abortions (RSA). NK-cells activity may be responsible for some cases of RSA. The objective of this study was to assess the role of the Th1 cytokines (IL-2, IFN- γ , TNF- α , TNF- β) in this phenomenon, and detection of Th1 cytokines in women with RSA. The control group consisted of 40 women with no history of pregnancy loss and complication. The abortion group comprised of 92 women having at least 3 pregnancy losses. Blood from the control group and women with RSA was taken at the end of first trimester of gestational age and at the time of abortion, respectively. Sera were separated and peripheral blood lymphocytes were challenged with PHA using RPMI medium. All samples were tested for concentration of Th1 cytokines using ELISA methods. It was considered that sera from women with RSA produced higher concentration of TNF- α , TNF- β , and IL-2 in comparison with sera from normal pregnant women (NPW) ($P < 0.001$). We were not able to detect IL-2 in (NPW) whereas in 31% sera from RSA group, IL-2 was detectable ($P < 0.001$). However, there was no significant difference between IFN- γ , concentration in sera from patients and control group ($P < 0.182$). Tissue culture supernatant from women with RSA also produced higher concentration of TNF- α , IFN- γ , TNF- β and IL2, than control group. These data may explain the increase NK-cells cytotoxicity during early pregnancy in women with a history of RSA. It may also provide a diagnostic tool to predict the outcome of pregnancy.

Keywords: Recurrent Spontaneous Abortion, cytokines, T-cells, Normal Pregnant women

INTRODUCTION

Recurrent spontaneous abortion (RSA) is a common complication in pregnancy. RSA is defined as two or more pregnancy losses before 20 weeks of gestation. It has been claimed that RSA occurs in 1% of women who are trying to have children (1,2).

Recently in order to detect the cause(s) of spontaneous abortion and provide better diagnostic tools, a number of tests have been introduced. These tests, e.g. hormonal testing, chromosomal analysis, hysterosonography and immunological evaluation provide a better understanding and some treatment options.

It has been suggested that successful pregnancy is associated with a T-helper 2 (Th2) type phenomenon. However, during pregnancy Th1 cytokine production is down-regulated. On the other hand, T cells responses are characterized by the presence of their relative cytokines; (IL-2, IL-12, TNF and IFN- γ) for Th1 cells, whereas a Th2 response is characterized by IL-4, IL - 5, IL-6 and IL-10 (3).

These and other cytokines which are low molecular weight proteins, play an integral role in the immune system of the human, e.g. T-cell mediated immunity, cancer, autoimmunity and allergy (4).

Th1/Th2 cytokine ratios are significantly elevated in women with RSA; hence Th1 cytokine production may be upregulated in these women(2,5).

Production of cytokines and the distribution of the immune cells during pregnancy may provide important evidences for predicting pregnancy outcome, eg, term or loss of pregnancy. Data provided by several investigators indicate that increased number of NK-cells is associated with RSA (5). Decidua during early pregnancy has NK-cells with distinctive phenotype eg, CD56 (+) CD16 (-)(5).

It has been shown that NK-cells proliferate and activate in response to IL-2. In the present study, IL-2 and other Th1 cytokines level in sera and supernatant from activated peripheral blood mononuclear cells (PBMC) culture during early pregnancy were measured in normal pregnancies, and compared with those from women with recurrent pregnancy loss.

MATERIALS AND METHODS

5 ml venous blood samples for measurement of various cytokines were taken from 92 women with RSA (having at least 3 pregnancy losses) and 40 normal pregnant women and sera were separated.

Peripheral blood was also layered on Ficoll-paque (pharmacia Biotech, Sweden), and peripheral blood mononuclear cells (PBMC) were separated, using density gradient centrifugation. PBMC were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCOBTL) containing 10% fetal calf serum (GIBCO). Cells were aliquoted into 96-well tissue culture plate at a density of 105 cells per well and then cells were subjected to stimulation using mitogen phytohemagglutinin (PHA) (sigma chemicals, St Louis MO. USA) at a concentration of 5 μ g/ml for a period of 96hr. Supernatants were harvested at 24 and 96 hr. As the major effect of cyclosporin A is to decrease IL-2 production, it was used as a control so as to check the stimulation.

Assessment of Th1 cytokine concentrations

Sera and supernatants concentrations of IL-2, IFN- γ , TNF- α , TNF- β were determined by enzyme-linked immunosorbent assay (ELISA). TNF- β Kit was obtained from R&D systems (Minneapolis, USA) and IL-2, IFN- γ , and TNF- α were obtained from Coulter/ Immunotech SA (France).

Manufacturers' protocols were followed for assessment; samples were added into wells in triplicate which suppose the relevant cytokine to be captured by monoclonal-anti-cytokine antibody bound to the well of the plate. Second biotinylated monoclonals as well as streptavidine - enzyme was added. Plates were washed after incubation period and chromogene substrate was added.

The intensity of coloration was detected using ELISA reader (Hyperion).

This is relative to the concentration of cytokine present in each sample.

Standard curves were plotted for each Th1 cytokine using reference cytokines and these curves were used for reading results.

The sensitivity for IL-2 was 5 pg/ml and for TNF- β , TNF- α , INF- γ were 7,10 and 3 pg/ml respectively.

Statistical Analysis

For statistical analysis, the non-parametric Mann-Whitney U test and Student t-test were used, for sera and tissue culture products, respectively.

Means and standard deviations (SD) are presented for describing variables with continuous distribution. Mean and proportion of various cytokine levels of cases and controls were compared using t-test, Mann-Whitney U tests and chi-square tests.

The odds ratio was used to estimate the ratio of the risk of RSA among patients with higher various cytokine level to the risk among NPW. The 95% confidence interval (CI) for OR was calculated using confields' method. All testing for statistical significance was two-tailed, and performed at $p < 0.05$.

RESULTS

Differences in distribution of age, gravidity and number of previous spontaneous abortions among 92 cases and 40 controls are shown in Table 1. Cases had more gravidity and number of previous spontaneous abortions than controls. The cases and controls were comparable with respect to age. The cases ranged in age from 22 to 35.6 years, with a mean (SD) of 26.7 (6.4) years. The controls ranged in age from 23.2 to 31.4 years, with a mean (SD) of 27.2 (5.1).

Concentration of TNF- α

A: Sera

In 61% of the sera of pregnant women, TNF- α was detectable, whereas a higher percentage of women with RSA showed TNF- α , (78%) (Table 2).

Comparison of TNF- α concentration between two groups indicated that in the RSA group this was significantly higher than normal pregnant women:

mean (SD) 125 (34) and 36 (16) respectively. $P < 0.001$ (Table 2).

B-Tissue culture supernatant

TNF- α were tested in supernatants obtained at 24 and 96 hr. In all supernatants sample, the levels of TNF- α were significantly higher by PBMC of the

RSA group than by control group ($P < 0.001$ for 24 and 96 hr) (Tables 3 and 5).

Concentration of IFN- γ

A:Sera

The presence of the IFN- γ was detectable in the sera of both groups, e.g. 42% incidence in the NPW and 54 % in the RSA group (Table 2). However comparison of the concentration between two groups showed no statistically significant difference [mean (SD)=22 (16) and 18 (15), respectively,] ($P = 0.182$).

B-Tissue culture supernatant

Levels of IFN- γ were determined at 24 and 96 hr. It was considered that higher concentration of IFN- γ produced by the PBMC of the RSA group than the cells of NPW ($P < 0.001$) (Tables 3 and 5).

Concentration of TNF- β

A:Sera

Levels of TNF- β were detectable only in 13 % of NPW and 22 % of sera of RSA. There was a statistically significant difference between two groups when the concentrations were compared.

(mean (SD)=58 (12) for RSA group and 45 (9) for control group). (Table2).

B.Tissue culture supernatant

Significantly higher levels of TNF- β were produced by PBMC RSA group compared with the control group ($P < 0.01$) (Tables 3 and 5).

Concentration of IL-2

A:Sera

IL-2 was not detectable in the sera of normal pregnant women. However, in 31% of sera from women with RSA, IL-2 was detectable (mean (SD)=67 (23) (Tables 3,4).

B=Tissue culture supernatal

Table 5 depicted that IL-2 level was detectable in these samples. A significant level of IL-2 was produced by PMBC from women with RSA compared with NPW both at the 24 and 96 hr ($P < 0.001$) (Tables 3, 4).

T-helper 1 cytokines increases and recurrent spontaneous abortion

Table 1. Group mean comparison for age, gravidity and number of previous spontaneous abortions between 92 recurrent spontaneous abortions and 40 normal pregnant women.

Characteristics	NPW N=40	RSA N=92	Difference (95 % CI)
	Mean (SD)	Mean (SD)	
Age (year)	27.2 (5.1)	26.7 (6.4)	0.5 (-1.8 to 2.8)
Gravidity	2.8 (1.3)	4.9 (1.7)	-2.1 (-2.7 to 1.5)*
No. of spontaneous abortion	0.0 (0.0)	4.7 (1.6)	4.7 (-5.1 to 4.1)*

* P<0.001. CI= confidence interval, RSA = recurrent spontaneous abortion
NPW= normal pregnant women.

Table 2. Comparison of various cytokine concentrations in serum among 90 recurrent spontaneous abortion and 40 normal pregnant women.

Cytokines	RSA Mean (SD)	NPW Mean (SD)	Difference (95 % CI)
IL-2	67 (23)	0.0(0.0)	67 (62.2 to 71.6)*
IFN- γ	22 (16)	18 (15)	4.0 (-1.9 to 9.9) **
TNF- β	58 (12)	45 (9)	13.0 (9.3 to 16.7) *
TNF- α	125 (34)	36 (16)	89 (80.4 to 97.6)*

* *P<0.001 , ** P<0.182 IC= confidence interval, RSA = recurrent spontaneous abortion, NPW= normal pregnant women.

Table 3. Comparison of various cytokine concentrations in tissue culture supernatant after 24 hours among 90 recurrent spontaneous abortions and 40 normal pregnant women.

Cytokines	RSA Mean (SD)	NPW Mean (SD)	Difference (95 % CI)
IL-2	1240 (125)	410 (31)	830(790 to 870)*
IFN- γ	2800 (223)	1620 (97)	1180(1107 to 1253)*
TNF- β	2610 (264)	1100 (80)	1510(1426 to 1594)*
TNF- α	8200 (710)	3720 (214)	4480(4253 to 4707)*

* P<0.001. IC= confidence interval, RSA = recurrent spontaneous abortion
NPW= normal pregnant women.

Table 4. Comparison of various cytokine concentrations in tissue culture supernatant after 96 hours among 90 recurrent spontaneous abortions and 40 normal pregnant women.

Cytokines	RSA Mean (SD)	NPW Mean (SD)	Difference (95 % CI)
IL-2	2840 (179)	835 (53)	2005 (1948 to 2062)*
IFN- γ	3930 (255)	2400 (184)	1530(1442 to 1618)*
TNF- β	10058 (689)	6310 (243)	3748(3527 to 3969)*
TNF- α	13500 (869)	5800 (249)	7700(7423 to 7977)*

* P<0.001. IC= confidence interval, RSA = recurrent spontaneous abortion
NPW= normal pregnant women.

Table 5. The risk of recurrent spontaneous abortion from various cytokines in serum.

Cytokines	RSA No.(%)	NPW No.(%)	Odds ratio (95 % CI)
IL-2	28 (31.0)	0.0(0.0)	17.1 (2.3 to 130.0) **
INF- γ	50 (54.3)	15 (37.5)	2.0 (0.9 to 4.2)
TNF- β	20 (21.7)	5 (12.5)	1.9(0.7 to 5.6)
TNF- α	72 (78.3)	58.00 (24.9)	2.4(1.1 to 5.4)*

* P<0.005. **P<0.001. IC= confidence interval. RSA = recurrent spontaneous abortion.

NPW= normal pregnant women.

Tables 2,3 and 4 show the cytokine ratio in the sera and tissue culture supernatant in the RSA group versus NPW. The overall consideration is that there is a higher concentration of Th1 cytokines in the sera and tissue culture supernatant well from women with RSA compared with the control group.

DISCUSSION

Although in recent years some progress has been made in better understanding of pregnancy loss and offering diagnostic tools and treatment procedures, still it remains the most common complication of pregnancy.

It seems that various tests may detect the cause(s) of recurrent spontaneous abortion. Chromosomal analysis, hormonal testing, uterus examination (hysterosonography) and immunological evaluation are examples of these evaluations. It is also known that determination of the miscarriage's causes may provide the appropriate treatment, e.g. immunotherapy may be used when immunological cause is thought. However, cytokines measurement is one of the well-known of these tests.

Cytokines measurement would provide an acceptable diagnosis in several human diseases, e.g. transplantation rejection (6), pregnancy (7,8) cancer (9), autoimmune diseases (10) allergy (4) etc. On the other hand, different cells may synthesize the relevant cytokines, e.g. INF- α , TNF- β , IL-2 for Th1, whereas Th2 - cells produce IL-4, IL-6, IL-10 (3) (11) (12). The effects of different cytokines also is well-known in some diseases: e.g. up-regulation of Th1-responses occur in preeclampsia (13).

In the present study, various Th1 cytokines have been measured to know their roles in miscarriages. The Th1 cytokines, INF- γ is supposed to contribute to embryo and trophoblast toxicity (14), but no statistically significant differences were found in two groups, however, in tissue culture supernatant, a higher concentration of INF- γ in women with RSA was considered in comparison with control group.

Sera concentration of other cytokines and tissue culture supernatant of all cytokines measured in this study were significantly higher in women with RSA than control group (Table 2).

The results of present investigation and data reported by Makhseed et al (15) show some similarities: e.g. in both studies, Th1 cytokines detected in sera of women with spontaneous recurrent abortion were significantly higher than normal pregnant women. However, in this study Th2 cytokines were not measured. Data presented in this study have shown that IL-2 was detected. Makhseed et al were unable to detect IL-2. The results obtained from Favier et al (16) and Mallmann et al (17) were in favour of Makhseed et al investigation. It is suggested that the cytokines measurement may provide a better understanding for different cytokines roles in pregnancy loss. Hence Th2-cytokines detection is highly recommended.

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T-helper 1 cytokines increases and recurrent spontaneous abortion

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