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Favorable immune phenotype predicts successful implantation and pregnancy

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ABSTRACT

Immune markers that may predict IVF failure and successful implantation and pregnancy were studied. Favorable immune parameters were selected based on 90% of data of women who got pregnant and had uneventful pregnancy course and outcome in present IVF cycle. Immune phenotype and NK cell activity of peripheral blood of 123 women with multiple IVF failure were studied by flow cytometry. Some parameters that were out of favorable borders (elevated expression of CD56, CD158a in T lymphocytes, decreased levels of CD4 T lymphocytes, up-regulated expression of HLA DR in CD8+ T cells and NK cells, elevated number of NK cells and increased NK cytotoxicity, increased and decreased expression of CD158a and CD8 in NK cells) were considered to be immune deviations (ID) potentially predictive for IVF failure. In women with 0–1 ID implantation rate (IR) was 50.9% (27/53), with two ID – 42.8% (12/28), with three and more ID – 21.4% (9/42). IR in group with three ID was lower than in group with 0–1 ID ($p < 0.01$, OR = 3.8, CI: 1.52–9.48) and in group with two ID ($p < 0.05$). Live birth rate (LBR) in women with 0–1 ID was 33.9%, with two ID – 28.5%, with three and more ID – 9.5%. LBR in group with three ID was lower than in group with 0–1 ID ($p < 0.01$, OR = 4.8, CI: 1.52–15.8) and in group with two ID ($p < 0.05$). The absence or single ID seems to be more favorable for successful IVF program. Combination of ID may predict implantation failure to a greater degree than isolated ID. Multiple immune deviations form unfavorable "immune phenotype" for implantation and pregnancy development.

1. Introduction

Implantation and pregnancy are dynamic, complex and highly coordinated processes involving systemic and local changes in maternal immune system. Natural killer (NK) cells are known to play a critical role in implantation. During the period of "receptive endometrium", "implantation window", decidualization of endometrium the number of dendritic and NK cells increases that is followed by implantation [1]. It has been confirmed that decidual NK cells are involved in the mediation of trophoblast invasion and modifying maternal spiral arteries to increase blood flow to the placenta [2,3]. In the first trimester of pregnancy NK cells, dendritic cells and macrophages infiltrate the decidua and accumulate around the invading trophoblast cells [4,5]. Maturation of uterine NK cells during pregnancy is critical for trophoblast invasion. In the absence of NK cells trophoblast cells are not able to reach the endometrial vascular system that may lead to termination of pregnancy [6]. Systemic immunity of pregnant women is not suppressed but modulated and pregnancy represents important period to protect the mother and the fetus [7].

In 1996, Beer et al. [8] determined higher levels of NK lymphocytes (CD56+, CD56+CD16+) in peripheral blood of non-pregnant and pregnant women with recurrent spontaneous abortions of unknown etiology than in fertile control and proposed elevated level of NK cells as a good prognostic marker of close pregnancy loss.

Wide implementation of IVF (in vitro fertilization) has solved many infertility problems. However, the number of women with IVF failure is growing, and in many of these cases standard IVF procedure does not lead to the desired pregnancy. Certain number of women with IVF failure was shown to have different impairments of systemic immunity parameters. Increased levels and

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enhanced activity of NK cells are most often reported [9]. The female body should be ready for establishing the domination of Th2 type immune response which accompanies pregnancy [10]. A strong predominance of Th1 immune response is observed in cases of multiple IVF failures. Activation and growth of peripheral blood T lymphocytes number with expressed activation markers on them lead to TNF-α-producing CD4+ level increase – the higher the levels that are observed, the more often IVF failure is registered [11]. IVF failures may be related to natural killers system (NK cells) – growth of NK cells levels and enhancing of their cytotoxicity [12]. NK cell receptors (KIR-receptors) suppress NK cells activity by balancing the activating and inhibitory receptors. This balance impairment is observed in spontaneous abortion and infertility [13]. The most reliable among KIR-receptors is CD158a because its expression is not associated with an individual HLA type. As anti-CD158a can recognize both inhibitory and activating KIRs, KIRs expression may be involved with increased or decreased cytotoxic function [14]. Elevated numbers of CD56+CD3+ NKT cells in infertile women were detected [15].

Association of peripheral NK cells and infertility was shown by some authors [16–20], but it was not confirmed by others [21]. Controversial results were received about association of uterine NK cells (uNK) and infertility. Some authors confirmed association of uNK and infertility [22–24], but others found no association [25,26]. As we mentioned earlier, IVF failures are also associated, in addition to enhanced NK cytotoxicity, with increased expression of CD8, CD158a and HLA DR in NK cells. Increased NK cytotoxicity alone, not accompanied by other impaired parameters, is not supposed to affect IVF success significantly [27].

The studies mentioned above demonstrated that immune parameters changes of women with IVF failures were not severe but presented as immune deviations and may be variable, multiple and sometimes even discrepant. It may be assumed that balancing of single and multiple immune deviations by systemic immunity may be different. In the present study we extended the spectrum of systemic immune parameters to evaluate a larger and wider cohort of patients with IVF failures. The aim of our work was to study immune criteria that may be predictive for successful implantation and pregnancy.

2. Materials and methods

The study was designed as a prospective cohort study. A total of 123 women (23–35 years old) with repeated implantation failures in IVF cycles (three or more) with good embryo quality and quantity (two embryos of high quality per each embryo transfer) who underwent in present IVF cycle in clinic of reproductive medicine "Nadiya" (Kyiv, Ukraine) were studied. The period of infertility was 7.1 ± 1.9 years. Tubal infertility was observed in 52% of patients. The patients enrolled did not have autoimmune diseases according to anamnesis and observation. Patients had no childbirth and spontaneous abortion. No chromosomal, endocrine, infectious factors and anatomic defects were observed. Women with male infertility factors were not included to the study. All of the subjects signed an informed consent prior to joining the study. The study was approved by the Ethics Committee of the Institute of Pediatrics, Obstetrics and Gynecology, National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine, in accordance with the Helsinki Declaration of 1975 on human experimentation.

A long luteal gonadotropin-releasing hormone (GnRH) agonist (*Diferelin*, *IpsenPharma*, *Paris*, *France*) protocol with controlled ovarian stimulation with recombinant follicle-stimulating hormone (*Gonal-F*, *Merk-Serono S.A.*, *Aubonne*, *Switzerland*) was used.

Peripheral blood samples were received from patients and were studied on days 17–23 of the menstrual cycle before gonadotropin stimulation in the IVF procedure. Clinical status of patients was studied before and during the pregnancy.

Lymphocyte subsets were studied by three-color flow cytometry in whole blood after staining with monoclonal antibodies conjugated with fluorochromes *FITC*, *PE*, *PE-Cy 5*; erythrocytes were lysed by lysing solution (reagents and equipment BD Biosciences, USA). Blood samples were analyzed with a FACScan flow cytometer. For determining the lymphocyte gate, the whole blood samples were incubated with mAbs of *LeucoGATE-CD45FITC/CD14PE kit* to permit discrimination of lymphocytes from granulocytes, monocytes, debris, and unlysed or nucleated red blood cells. In addition, the whole blood samples were incubated with SimulTEST control (*IgG1-FITC + IgG2-PE*) as a background control. For normal ranges of the main lymphocyte subsets, we referred to the recommendations of *BD Biosciences* [28].

Flow cytometry was used for measuring of peripheral blood lymphocyte cytotoxic activity, using a FACScan flow cytometer. We stained K562 target cells with fluorescent CFDA (carboxy-2,7-dichlorofluorescein diacetate) (Mol Probes, USA). Effectors (peripheral blood mononuclear cells) were purified on a Histopaque™-1077 density gradient ("Sigma", USA). Target and effector cells were incubated at ratios of 1/10 and 1/20 for 4 h in RPMI-1640 with 10% bovine serum in an incubator with 37 °C with 5% CO_2 ("Revco", Switzerland). Then, cells were stained with propidium iodine (PI) ("Sigma", USA), which stains dead (permeabilized) cells. After measurement of PI+ CFDA+ cell level with the FACScan, the cytotoxicity index was calculated for every ratio effector/target by the following formula: % cytotoxicity = [%PI+ cells (dead target cells)/%CFDA+ cells (whole target cell number)] × 100%.

Potential "candidates" for immune deviations were selected during long period of routine immune investigation of patients in IVF program. The main demands were higher frequency of abnormal indices in patients with unsuccessful IVF program if to compare with IVF success in subsequent IVF cycle, stability of specific parameters in repeated measuring during some months, sufficient methodical reproducibility and accuracy. We selected 12 parameters of conditional immune deviations from the "borders of IVF fertility", which were formed by 90% of data of selected immune indices of 18 women with IVF failure in anamnesis but who got pregnant and who had uneventful pregnancy course and outcome in present IVF cycle (actually fertile women) [27]. Divergences of the parameter out of 90% "corridor" in women with successful pregnancy were registered as an immune deviations or "immune accentuations", because these deviations were not very strong and considerable as in severe disease. In this work we accepted immune deviations presented in Table 1 as significant and stable: Elevated NK cytotoxicity (>30% of NK cytotoxicity at ratio target/effector cells 1:10 and >40% of NK cytotoxicity at ratio target/effector cells 1:20); CD56+ in T cells (CD3+) > 18%; CD158a+ in T cells (CD3+) > 5%; CD4+ T cells (CD3+) < 40%; HLA DR+ in CD8+ T cells > 30%; NK cells (CD56+CD3−) > 21%; HLA DR+ in NK cells (CD56+CD3−) > 25%; CD8+ in NK cells (CD56+CD3−) > 64%; CD8+ in NK cells (CD56+CD3−) < 40%; CD158a+ in NK cells (CD56+CD3−) > 65%; CD158a+ in NK cells (CD56+CD3−) < 17%. Representative frequency histograms of immune tests in women with successful implantation and women with IVF failures are presented in Supplementary Figs. S1 and S2. In the present work we included the most simple, stable and frequent immune deviations to obtain a wider group of women with IVF failure. Some other parameters that also seemed to be important but not stable enough from cycle to cycle according to our previous study were not included to the study (CXCR3, CCR4 in T lymphocyte and NK cells, intracellular cytokines IFN-gamma/IL10 in CD4+ T lymphocytes, T reg cells, CD95 and CD69 in T lymphocyte and NK cells).

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