THEORY OF IMMUNE ACCENTUATIONS:
FORMATION OF ACCENTUATED IMMUNOPHENOTYPE

Abstract. In numerous previous studies, we have formulated the idea of immune accentuation, demonstrated associations between immune accentuations and the unfavorable course of physiological processes, and shown that the association of several accentuations has a reliable negative effect on the further course of the reproductive process. In the article, we demonstrated the correlative and associative nature of certain immune accentuations, which explains the causes of the formation of an unfavorable immuno-accentuated phenotype. Thus, we found highly reliable correlative and associative relationships between the parameters of natural resistance (NK quantity, NK cytotoxicity, expression of CD158a and CD8 on NK cells), all these parameters were mutually correlated with each other and their accentuations were associated with accentuations of partner parameters. Another immuno-accented cluster had pro-inflammatory features, so the expression of HLA-DR on NK and T-cytotoxic cells, as well as NKT-like populations (CD3+CD56+ and CD3+CD158a+) also had highly reliable correlations and associations. This pro-inflammatory phenotype was negatively correlated and inversely associated with T-helper levels. Presumably, this is a consequence of uncontrolled chronic activation with the appearance of alternative ways of differentiation of T cells that bypass regulatory control by T-helpers. We showed a fundamental difference in the association and correlative relations between immune parameters depending on their level. Thus, HLA-DR levels on NK cells within (NK HLADR < 24 %) had a negative correlation with % NK cells, while, within (NK HLADR > 24 %) they begin to correlate positively and reliably with the number of NK. So, accentuation is the place in the distribution of the parameter, where the parameter begins to put pressure on other parameters and determine their level of direction. These are the same zones where the parameter becomes unfavorable, because it loses the possibility of regulation. Accentuation of the immune parameter is associated with specific accentuations of other parameters. Whether it causes changes in other links or whether it and other changes are the result of extra-immune or environmental factors are unknown. But it is clear that this leads to the formation of an immuno-accented phenotype. Accentuation creates prerequisites for the formation of other immune accentuations due to the imbalance of systemic immune regulation.

Keywords: Immune accentuations; IVF failures; Reproductive failures; NK cytotoxicity; HLA-DR expression; NK lymphocytes.

INTRODUCTION

“Accentuation” is a term that was proposed in psychiatry by K. Leonhard [1] and quite successfully illustrates a certain feature that is an absolutely normal option, but can become an unfavorable
factor for the course of certain processes. We proposed an immunological interpretation of this term 10 years ago. “Immune accentuation” is such a state of a certain link of the immune system that is not pathological, but can become unfavorable for certain physiological processes or environmental challenges. At the same time, the accentuation itself can be a favorable factor for the course of another physiological process. Any specialization of the immune system makes it more effective for performing specific functions for which this specialization is formed and less effective for performing another function for which this specialization is not adapted. The genetic basis of this idea is well covered in the works [2]. Same authors also explain that different combinations of maternal KIR and fetal HLA-C variants are correlated with low birth weight and pre-eclampsia or high birth weight and obstructed labor, the two extremes of the obstetric dilemma [3].

Same dilemma was shown in KIR/HLA-C combinations that protects against malaria and at same time creates a high risk of pre-eclampsia [4]. A universal combination does not give special benefits, but also does not cause special risks. Researching immune accentuations for 10 years, we have described a number of such that lead to reproductive losses [5]. NK cytotoxicity that is too high or low is an unfavorable factor for embryo implantation and the course of pregnancy [6]. As well as too high and too low expression of CD8 [7], CD158a [8], and CD335 [9, 10] on peripheral blood NK lymphocytes associated with reproductive failures. Elevated or reduced NK cell activation against target is also an unfavorable factor in the prognosis of the reproductive process [11, 12].

High levels of immune cell activation HLA-DR expression on T and NK cells is a factor of an unfavorable prognosis in reproduction [13, 14] and similar levels are a factor of an unfavorable state of health during adaptation among participants of the Antarctic expedition [15]. But reduced activation of NK-lymphocytes may contribute to increased susceptibility to viral infections and bacterial complications [16].

That is, the accented parameter creates difficulties for adapting the system to new challenges. We separately investigated different NK cytotoxicity and levels of NK cells and found that the zones where these factors are unfavorable (too high and too low) are precisely the zones where these parameters are highly reliably correlated with each other [17] and in the reproductively favorable zone there is no correlation between them [18]. That is, in favorable zones, the number of NK cells does not determine the function of NK cells. In this zone, the function can be adjusted according to actual needs (new challenges, whether it is an embryo or a viral infection), but in the accented zones, the quantity directly determines the function, and it is already very difficult to adjust the function with such an accented quantity [19].

But we also found that an isolated single accentuation has a negligible effect on the further reproductive process, while the combination of several (systematic accentuations) has a highly reliable negative effect on the further success of the reproductive process. That is, the combination of accentuations of immune parameters multiplies their negative impact [13, 14].

In this study, we conducted an associative and correlative analysis of immune parameters on a large group of patients with reproductive problems.

METHODS

Infertile patients (n = 1554) were selected for this study. Patients with idiopathic in vitro fertilization (IVF) failures in anamnesis (> 2), was younger than 39 years old, were negative for anticardiolipin antibodies, with normal karyotype and without endometrial insufficiency and thrombotic disorders. Patients did not obtain any additional treatment and not have actual infections.
We analyzed lymphocyte phenotype as described previously [20] using a FACScan flow cytometer (BD Bioscience, San Jose, USA) equipped with CellQuest software. Patients were tested on anticardiolipin (aCL) IgG antibody as described [21, 22]. NK cell cytotoxicity was measured as described previously [23] using a FACScan flow cytometer (BD Bioscience, San Jose, USA) equipped with CellQuest software.

**STATISTICAL ANALYSIS**

The statistical analysis of the results was performed using Fisher’s Exact Test (unpaired, non-parametric, two-sided P value) and the Spearman and Pearson correlations (In Stat version 3.0 for Windows Graph Pad Software Inc., San Diego, CA, USA).

**RESULTS**

A number of multiple correlations were found between immune populations (Fig. 1). We did not notice a correlation, which is the result of a mathematical calculation of the % of cells. Of course, % NK cells will be inversely correlated with % T both Th and Tc because the percentages are only 100 and the more T, the less NK of course. In Fig.1 we shows only physiological correlations. Thus, we found a highly reliable positive correlation between the number of NK cells, NK cytotoxicity and the level of CD8 and CD158a on NK. All these parameters correlated with high reliability and formed an isolated intercorrelated core. The level of NK cells and the level of CD8 on NK cells were inversely correlated with the level of HLA-DR on NK cells. The level of HLA-DR on NK cells is included in another correlative cluster that united HLA -DR on T cells and CD56 and CD158 on T cells. All these parameters correlated with each other and, as a rule, did not correlate with the first cluster. Only the expression of CD56 and CD158a on T cells was correlated with the level of CD158a on NK. And HLA DR on T cells with the level of NK. This pro-inflammatory cluster was negatively correlated with T-helper levels. Thus, according to intercorrelations, a cluster of natural resistance was distinguished, which included the number and cytotoxic activity of NK and the differentiation of NK cells by CD8 and CD158a. A separate cluster was pro-inflammatory, which included the expression of the activation marker HLA-DR on NK and T lymphocytes, as well as the levels of NKT-like cells CD3+CD56+ and CD3+CD158a+. The level of T-helpers appeared as a completely separate parameter that had only a negative correlation with pro-inflammatory parameters (Fig. 1).

**ZONAL CORRELATION**

Analyzing the correlation ratios, we found their dependence on the parameter level. The most striking example of such dependence was the correlation between the level of HLA-DR on NK and the total level of NK lymphocytes. Thus, for the levels of HLA-DR on NK (< 24 %), the correlation with the NK % was reliably positive (Fig. 2). However, it became reliably positive for HLA-DR levels on NK (> 24 %). That is, in a pro-inflammatory state, the correlation is positive, and in a calm state, it becomes negative. Thus, at high levels of HLADR on NK, they increase in parallel with the number of NK. However, when HLADR expression on NK is not increased, it is conversely associated with decreased NK. This is an extremely important result also because the point where the correlation curve fundamentally changes its character (24 % HLADR on NK) is identical to the beginning of accentuation of this parameter (unfavorable for reproduction).
Fig. 1. Correlation networks between immune populations (n = 1554)

The arrows show the parameters with which the correlation analysis was performed. Correlation coefficient (r) Linear Regression is indicated above the arrows. The P value is indicated under the arrow.

Red arrows for positive correlations, green for inverse.

NK cytotox- NK cytotoxicity. NKCD8 - % expression of CD8 on NK (CD3-CD56). NKCD158a - % expression of CD158a on NK (CD3-CD56). DR on NK - % expression of HLA-DR on NK (CD3-CD56).

%NK- frequency of NK (CD3-CD56+) from all lymphocytes. Tc HLA-DR - % expression of HLA-DR on T-cytotoxic lymphocytes (CD3+CD8+). T CD158- % expression of CD158a on T-lymphocytes (CD3+).

Fig. 2. Correlation between NK frequency and expression HLA-DR on NK cells
We showed an example of complex correlative relationships between parameters. At the levels of HLA-DR expression in NK lymphocytes (> 24%), this parameter is reliably correlated with the total percentage of NK, while at the levels HLA-DR expression in NK lymphocytes (< 24%), it begins to be reliably inversely correlated with the level of NK. Association of accentuations

If the correlative relationships can change from positive to negative under different states of the parameter, then it is more correct to compare associations between accents zone. So we analyzed the associativity between them. If accentuation A is present, with what probability is it associated with accentuation B compared to unaccentuated states. Thus, we discovered a whole series of associations between accentuations in (Fig. 3).

Fig. 3. Associations networks between immune accentuations (n = 1554)

Arrows show significant relationships between accentuated parameters. OR → is indicated from the top from accentuation A to accentuation B. OR ← from accentuation B to accentuation A.

NK cytotox- NK cytotoxicity (red > 40 at E/T ratio 20/1. blue < 10 at E/T ratio 20/1). NKCD8 - % expression of CD8 on NK (CD3-CD56) (red > 60 %, blue < 40 %). NKCD158a - % expression of CD158a on NK (CD3-CD56) (red > 60 %, blue < 40 %). DR on NK - % expression of HLA-DR on NK (CD3-CD56) (red > 24 %). % NK- frequency of NK (CD3-CD56+) from all lymphocytes(red > 18 %. blue < 5.5 %). Tc HLA-DR - % expression of HLA-DR on T-cytotoxic lymphocytes (CD3+CD8+)(red > 34 %). T CD158- % expression of CD158a on T-lymphocytes (CD3+CD8+)(red > 5 %). T CD56 - % expression of CD56 on T- lymphocytes (CD3+)(red > 15 %). Th-frequency of T-helpers (CD3+CD4+) from all lymphocytes (blue < 40 %).

Naturally, high levels of NK cells were associated with high levels of NK cytotoxicity, and low NK levels with low cytotoxicity. Likewise, high levels of NK cytotoxicity and NK cells were associated with high expression of CD8 on NK cells. In turn, high levels of CD8 on NK cells were associated with high levels of CD158a on NK cells. As in correlation studies, high natural resistance forms a mutually associated cluster. It’s a possible that high differentiated (mature) NK phenotype associated with increase CD8 and CD158a expression and increased NKc (accentuated natural resistance phenotype).
However, a high level of NK cells also correlated with a high expression of HLA-DR on T cells. That is, the inflammatory phenotype also affects the level of natural resistance. High expression of HLA-DR on T cells was associated with reduced levels of CD8 on NK cells and with simultaneously high and low levels of CD158a on NK cells (pro-inflammatory modify natural resistance phenotype).

The pro-inflammatory phenotype (high levels of HLA-DR on T and NK cells and high levels of CD56 and CD158a on T cells had a highly reliable reciprocal association) So all these changes are from the same source. Also, this immunophenotype (high levels of HLA-DR on NK cells and high levels of CD56 and CD158a on T cells) had a reliable association with a markedly reduced level of T-helpers. It's possible that a reduction of Th regulation result to accumulation of NKT-like forms of T lymphocyte with uncontrolled activation.

**CONCLUSIONS**

Well, of course, the most debatable issue is the question of the importance of immune populations of peripheral blood on embryo implantation processes. Where is the blood and where is the endometrium? But in fact, despite the dissimilarity of the immunophenotype of blood and endometrium, they have many common features. And it is the pro-inflammatory state in the peripheral blood that is reflected in the endometrium as well. Also, the high differentiated state of NK in the peripheral blood represents the same changes in the endometrium [24, 25].

1. Correlation between immune populations highlights important clusters with high correlation between parameters.
2. Correlations may have opposite values in different zones of the parameter.
3. Accentuated parameters are associated with accents of other parameters belonging to a similar associative group.

**NK cytotox** – NK cytotoxicity levels (%) at E/T ratios (15/1) (PBMC/K562). **NK CD8** – expression of CD8 on NK lymphocytes (%). **NKCD158a** – expression of CD158a on NK lymphocytes (%). **NK % levels** of NK (CD3-CD56+) from all lymphocytes. **DR on NK** – expression of HLA-DR on NK lymphocytes (%). **Tc HLA-DR** – expression of HLA-DR on T-cytotoxic lymphocytes (CD3+CD8+) (%). **T CD56** – expression of CD56 on T-lymphocytes (CD3+CD8+) (%). **T CD158a** – expression of CD158a on T-lymphocytes (CD3+CD8+) (%). (Pearson correlations $r =$ on top of an arrow, $p =$ two-sided P value)

At levels of HLA-DR expression on NK more than 24 %, the correlation between % NK and HLA-DR expression on NK was significantly positive ($r =$ 0.1955 $p = 0.0017$. In contrast, we found that at HLA-DR expression levels on NK less than 24 %, the correlation between % NK and HLA-DR expression on NK was significantly negative ($r =$ -0.2026).

**NK cytotox** – NK cytotoxicity (red > 40 at E/T ratio 20/1. blue <10 at E/T ratio 20/1). **NKCD8** – % expression of CD8 on NK (CD3-CD56) (red > 60 %. blue <40%). **NKCD158a** – % expression of CD158a on NK (CD3-CD56) (red > 60 %. blue <20 %). **DR on NK** – % expression of HLA-DR on NK (CD3-CD56) (red > 24 %). **NK frequency of NK** (CD3-CD56+) from all lymphocytes (red > 18 %. blue <5.5 %). **Tc HLA-DR** – % expression of HLA-DR on T-cytotoxic lymphocytes (CD3+CD8+) (red > 34 %). **T CD158** – % expression of CD158a on T-lymphocytes (CD3+) (red > 5,5 %). **T CD56** – % expression of CD56 on T-lymphocytes (CD3+) (red > 20 %). **Th frequency of T-helpers** (CD3+CD4+) from all lymphocytes (blue < 40 %).

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Б. В. Донський, кандидат біологічних наук,
ORCID ID: 0000-0001-9434-2826, Scopus Author ID: 12782751800, Google Scholar, Researchgate, НБУВ ID: 0097127,
Інститут педіатрії, акушерства і гінекології імені академіка О. М. Лук’янової НАМН України
https://ipag-kiev.org.ua, Київ, Україна
Лабораторія імунології https://ipag-kiev.org.ua/laboratoriya-imunologiyi,
boris_donskoy@ukr.net

ТЕОРІЯ ІМУННИХ АКЦЕНТУАЦІЙ:
ЯК ФОРМУЄТЬСЯ НЕСПРИЯТЛИВИЙ ІМУНОАКЦЕНТУВАНИЙ ФЕНОТИП

Анотація. У численних попередніх дослідженнях нами було сформульовано ідею імунної акцентуації, продемонстровано асоціації між імунними акцентуаціями та несприятливим
перебігом фізіологічних процесів та показано, що асоціація декількох акцентуацій має достовірний негативний вплив на подальший перебіг репродуктивного процесу. В статті ми продемонстрували корелятивність та асоціативність певних імунних акцентуацій, що пояснює причини формування несприятlivого імуноакцентований фенотипу. Так ми виявили високу достовірну кореляцію та асоціативну зв'язки між параметрами природньої резистентності (НК кількості, НК цитотоксичність, експресія CD158a та CD8 на НК клітинах), всі ці параметри взаємо кореляють між собою та із іншими акцентуаціями асоціювалися із акцентуаціями партнерських ланок. Інший імунноакцентований кластер мав про-запальні ознаки, так єкспресія HLA-DR на НК та T-цитотоксичних клітинах а також HKT-подібні популяції (CD3+CD56+ та CD3+CD158a+) також мали висок достовірні кореляції та асоціації. Такий про-запальний фенотип мав негативну кореляцію та зворотною асоціацію із рівнями Т-хелперів. Імовірно це і є наслідком неконтрольованої хронічної активації із появою альтернативних шляхів диференціації Т клітин, що обходять регуляторний контроль з боку Т-хелперів. Ми показали принципову різницю в кореляції та корелятивних відносин між імунними параметрами залежно від їх рівня. Так рівні HLA-DR на НК клітинах у межах (НК < 24 %) мали негативну кореляцію із % НК клітин, тоді як, у межах (НК > 24 %) вони починають позитивно достовірні корелятувати із кількістю НК. Тож, акцентуація, це те місце у розподілі параметру, де параметр починає тиснути на інші параметри і визначати їх рівень напряму. Це ті самі зони, де параметр стає несприятливим, бо він втрачає можливість до регуляції. Акцентуація імунного параметру асоціюється із специфічними акцентуаціями інших параметрів. Чи вона викликає зміни в інших ланках чи вона і зміни є результатом дії позаклітинних або зовнішніх чинників невідомо. Але зрозуміло, що це приводить до формування імуноакцентований фенотипу. Акцентуація створює передумови для формування інших імунних акцентуацій через розбалансування системної імунної регуляції.

**Ключові слова:** імунні акцентуації; IVF втрати; репродуктивні втрати; НК цитотоксичність; Експресія HLA-DR; НК-лімфоцити.

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