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ИЗМЕНЕНИЕ ЭКСПРЕССИИ HLA-DR НА СУБПОПУЛЯЦИЯХ ЛИМФОЦИТОВ СУПРУГОВ, ИМЕЮЩИХ ДЕТЕЙ СО СПОРАДИЧЕСКИМИ ВРОЖДЕННЫМИ ПОРОКАМИ СЕРДЦА БЕЗ ХРОМОСОМНЫХ ЗАБОЛЕВАНИЙ, ПОД ВОЗДЕЙСТВИЕМ ЖЕНСКОЙ АУТОСЫВОРОТКИ

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Резюме. Целью настоящего исследования было изучение влияния женской аутосыворотки на экспрессию HLA-DR в различных субпопуляциях лимфоцитов супругов, имеющих детей со спорадическими врожденными пороками сердца без хромосомных заболеваний. В группу исследования включено 78 семейных пар, имеющих детей с врожденным порок сердца. Контрольная группа включала в себя 35 семейных пар, имеющих здоровых детей. Иммунный ответ в смешанной культуре лимфоцитов (СКЛ) супругов оценивали по увеличению экспрессии HLA-DR в смешанной культуре по отношению к спонтанным культурам лимфоцитов. Первичная окраска женских и мужских лимфоцитов моноклональными антителами к CD45, конъюгированными с различными флуоресцентными красителями (PC-5 и PC-7), позволила оценить иммунный ответ женских лимфоцитов на мужские и наоборот. Активирующий эффект женской аутосыворотки на все субпопуляции женских лимфоцитов одновременно встречался значимо реже в основной группе, по отношению к контролю. В группе контроля доминировал положительный эффект женской аутосыворотки на экспрессию HLA-DR для всех женских субпопуляций лимфоцитов одновременно. Для всех женских лимфоцитов, имеющих на своей мембране молекулу HLA-DR, в основной группе значимо чаще встречался блокирующий эффект женской аутосыворотки, по отношению к контролю. Таким образом, можно говорить о том, что эффект женской аутосыворотки проявляется по отношению к экспрессии HLA-DR на собственных лимфоцитах, но не на лимфоцитах супруга.

Ключевые слова: врожденные пороки сердца, экспрессия HLA-DR, лимфоциты, женская аутосыворотка

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CHANGES IN THE EXPRESSION OF HLA-DR ON LYMPHOCYTE SUBPOPULATIONS OF SPOUSES HAVING CHILDREN WITH SPORADIC CONGENITAL HEART DEFECTS WITHOUT CHROMOSOMAL DISEASES, UNDER THE INFLUENCE OF FEMALE'S AUTOSERUM

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Abstract. This study is aimed to investigate the effect of female autoserum on the HLA-DR expression in various subpopulations of lymphocytes obtained from spouses with children with sporadic congenital heart defects without chromosomal diseases. 78 married couples with children with congenital heart disease were included in the study group. The control group was formed from 35 married couples with healthy children. The immune response in a mixed culture of lymphocytes of spouses was evaluated by an increased HLA-DR expression in a mixed culture in relation to spontaneous cultures of lymphocytes. Primary staining of female and male lymphocytes by monoclonal antibodies to CD45 conjugated with various fluorescent dyes (PC-5 and PC-7) was performed to assess the immune response of female lymphocytes to male ones and vice versa. The activating effect of female autoserum on all subpopulations of female lymphocytes simultaneously occurred significantly less frequently in the study group compared to the control. The control group was characterized by the domination of the positive effect of female autoserum on HLA-DR expression for all subpopulations of female lymphocyte. For all female lymphocytes having HLA-DR molecule on its membrane, the blocking effect of female autoserum in the study group was significantly more expressed in relation to the control group. Thus, the effect of female autoserum is manifested in relation to the HLA-DR expression on its own lymphocytes, but not on the lymphocytes of the spouse.

Keywords. congenital heart defects, HLA-DR expression, lymphocytes, female autoserum

Introduction

Congenital heart defects (CHD) are one of the most common fetal malformations worldwide. It is occurring in 4-50 cases per 1000 newborns. CHD etiology and pathogenesis are triggered by mutually influencing exo- and endogenous factors. The formation of CHD starts in the embryonic period from the second to the eighth week of gestation. During this period of ontogenesis, the maternal immune microenvironment interacting with a semi-allogeneic embryo resulting in activation of various subpopulations of female lymphocytes, which can activate or limit the immune inflammation at the mother-embryo system. Regulating humoral factors dissolved in the female's autoserum additionally affect cellular interactions. Antibodies to native HLA-DR molecules presenting embryo alloantigens to T-lymphocytes with regulatory potential can be important humoral factors in female's autoserum limiting immune inflammation in the mother embryo-system. These antibodies affect the limitation or activation of immune inflammation in the mother-embryo system through this mechanism. Accordingly, the synthesis of pro-inflammatory cytokines by maternal immunocompetent cells will increase during decompensation of inflammation in the mother-embryo system. The proliferation and differentiation of the cells of upcoming cardiovascular system may be impaired by pro-inflammatory cytokines-activated pyroptosis. Finally, defects and abnormalities of the cardiovascular system can be formed. Since this stage of CHD pathogenesis is poorly studied, the aim of this research was to investigate the effect of female's autoserum on HLA-DR expression in various subpopulations of lymphocytes of spouses having children with sporadic CHD without chromosomal diseases.

Materials and methods

In the main group parents (n = 78 married couples) who have children with sporadic CHD without chromosomal diseases were included. All children were treated in the Department of Pediatric Cardiology, L. Barbarash Kemerovo Regional Clinical Car-

diology Center (Kemerovo, Russia). The diagnosis of CHD was confirmed by echocardiography and medical records. CHD in children was represented by the following nosologies: atrial septal defect occurred (22 children), interventricular septal defect (34 children), Fallot tetrad (6 children), pulmonary valve stenosis (5 children), partial abnormal pulmonary venous drainage (4 children), aortic coarctation (3 children), hypoplasia aortic arches (2 children) and defect in the aorto-pulmonary septum (2 children). There was no family history of CHD in the main group. In the control group 35 married couples with two or more healthy children, no reproductive loss and children with CHD were included.

The influence of female's autoserum on HLA-DR expression in various subpopulations of spouses' lymphocytes was studied on female and male monocultures. Lymphocytes were isolated from peripheral blood at a density gradient of 1.077 (Ficoll-1077, Dia-M, Moscow, Russia). The obtained suspensions of female and male lymphocytes were washed twice with Hanks solution (Dia-M, Moscow, Russia). 1000 μL of Hanks solution was added to the tubes with female's and male's lymphocytes and they were centrifuged for 10 min at 1500 rpm. Then supernatant was removed, female's lymphocytes were incubated with monoclonal antibodies conjugated with peridinin-chlorophyll 7 (PC-7) fluorescent dye and male's lymphocytes were incubated with peridinin-chlorophyll 5 (PC-5) (Biolegend, USA) for 15 min at room temperature in the dark. After incubation, the lymphocytes were washed from unbound antibodies with Hanks solution. The 2000 washed female's and male's lymphocytes were transferred into two plastic tubes for flow cytofluorimetry (Beckman Coulter, USA) and 1 μl of complete medium RPMI-1640 with 15% fetal calf serum (ETS, Gibco, Thermo Fisher Scientific, USA), 2 mM of L-glutamine (Panreac, Spain), 10 mM of Hepes buffer (Sigma, USA), 5×10^{-5} M of 2-mercaptoethanol (Biochem, France) and 50 μg/mL gentamicin solution sulfate (Vetinterpharm, Russia) were added into each tube. The first tubes with female's and male's lymphocytes were used as a control. In the second tubes with female's and male's lymphocytes, female autoserum was additionally added at the rate of 20% per suspension volume. All tubes were placed in a CO2 incubator for 2 h at 37 °C. After the incubation, lymphocytes from each tube was washed by Hanks solution according to the described above method followed by staining of each monoculture both in complete medium and in medium supplemented with 20% female autoserum using conjugated monoclonal antibodies (fluoriscein isothiocyanate (FITC) with CD3 and phycoerythrin (PE) with HLA-DR) (Biolegend, USA). 5 μL of each monoclonal antibody was added into all tubes. The volume of antibodies to the number of lymphocytes, the time and temperature of incubation were in accordance with the attached instructions for each conjugated monoclonal antibody. Incubation was carried out for 15 min at room temperature in the dark. Then, lymphocytes were washed with Hanks solution according to the described above method. 300 μL per tube of OptiLyse solution (Beckman Coulter, USA) was added to fix antibodies on lymphocytes in monocultures. The HLA-DR expression on various subpopulations of spouses lymphocytes and the effect of female's autoserum on this process were evaluated using flow cytometry protocol. The protocol included several sequential steps for each female and male monoculture incubated in complete medium and in medium supplemented with 20% female autoserum. The first stage was associated with the selection of the lymphocyte population in the first histogram according to their size characteristics (forward (small angle) light scattering – forward scatter – FSL) and intracellular density (lateral light scattering – side scatter – SSL). In the following stage, the lymphocyte pool was additionally clustered by the total leukocyte marker CD45 (for female monoculture – CD45-PC7; for male monoculture – CD45-PC5) and intracellular density (SSL). The third stage was the main one for this study. It is presenting how the isolated lymphocytes were analyzed by phenotypes: $CD3^+/HLADR^+$, $CD3^-/HLADR^+$, as well as $CD45^+/HLADR^+$. The analysis of these subpopulations was carried out in female and male monocultures both in the complete medium and in medium supplemented with 20% of female autoserum. In case of differential expression of HLA-DR on different subpopulations of female's and male's lymphocytes in complete medium and in a medium supplemented with female autoserum, its effect was determined. So, if the expression of HLA-DR on the corresponding subpopulation of lymphocytes in the environment with 20% female autoserum was lower than in complete medium, the effect of female autoserum was considered to be blocking. If the expression of HLA-DR on the corresponding subpopulation of lymphocytes in an environment with 20% of female autoserum was higher than in complete medium, the effect of female autoserum was considered to be activating. No differences in HLA-DR expression on the corresponding subpopulation of lymphocytes in the compared media was taken as the absent effect. Accordingly to these estimates, couples with different types and combinations of the effects of female autoserum on the expression of HLA-DR in various subpopulations of female and male lymphocytes were identified in the main and control groups.

Statistical analysis of the obtained results was carried out using the software package Statistica 10.0. Pairwise comparing the frequency of occurrence of various effects of female autoserum was performed using the non-parametric chi-square test calculating which the Yeats correction for the continuity of a small sample. For significant differences in indicators when comparing them in pairs in the main and

control groups, the odds ratio (OR) was calculated. Results were considered significant with a confidence error of less than 5%.

Results

The study showed that the blocking, activating and absent effects on HLA-DR expression in various subpopulations of lymphocytes did not significantly differ between female and male monocultures. This has been demonstrated both for individual subpopulations and for their combinations.

A comparative analysis of the combined effects of female autoserum with respect to HLA-DR expression for all subpopulations of female's and male's lymphocytes (Table 1) showed the only significant difference between the main and control groups. Thus, the activating effect of female autoserum on all subpopulations of female's lymphocytes was significantly less in the main group compared to the control. The control group was dominated by the positive effect of female autoserum on HLA-DR expression for all female lymphocyte subpopulations simultaneously.

An analysis of the effects of female autoserum on individual subpopulations of lymphocytes of female and male monocultures in the experimental and control groups is presented in table 2. According to Table 2, in the main group female autoserum significantly more often exerted a blocking effect on the expression of HLA-DR in a subpopulation of female lymphocytes $HLA\text{-}DR^+/CD3^-$ than in the control. In the control group for this subpopulation of female lymphocytes, the activating effect of female autoserum to HLA-DR expression was dominated.

Moreover, for all female's lymphocytes that have an HLA-DR molecule on their membrane, the blocking effect of the female autoserum in relation to the control was significantly more common in the main group. The direct opposite was the control group, where the activating effect of female autoserum to HLA-DR expression on female lymphocytes was significantly dominated. According to Tables 1 and 2, the main and control groups did not differ in the effect of female autoserum on the expression of HLA-DR on male lymphocytes.

Thus, we can say that the effect of female autoserum is manifested in relation to the expression of HLA-DR on own lymphocytes, but not on the spouse's lymphocytes.

Discussion

The obtained effect of female autoserum on HLA-DR expression in female and male lymphocyte subpopulations can be interpreted from several positions. The blocking effect can be formed due to female antibodies against their own HLA-DR molecules. And although the presented approach to study the influence of female autoserum on HLA-DR expression is not a variant of the method for evaluating antibodies to HLA-DR using the crossmatch methodology, it is still likely to identify antibodies to the HLA-DR molecule. Thus, antibodies dissolved in female autoserum could compete for HLA-DR with the corresponding monoclonal antibodies. Through this phenomenon a blocking effect could occur, because the number of $HLA\text{-}DR^+$ cells in suspension with a medium supplied with 20% female autoserum is de-

TABLE 1. COMPARATIVE ANALYSIS OF THE COMBINED EFFECTS OF FEMALE AUTOSERUM IN RELATION TO HLA-DR EXPRESSION FOR ALL SUBPOPULATIONS OF FEMALE'S AND MALE'S LYMPHOCYTES IN THE COMPARED GROUPS (DATA ARE PRESENTED IN ABSOLUTE VALUES)

Effects	Case n = 78		Control n = 32		Chi square (OR; p)
	Presence of effect	Lack of effect	Presence of effect	Lack of effect	
Blocking effect on all lymphocyte subpopulations in female and male monocultures	5	73	4	28	p > 0.05
Activating effect on all lymphocyte subpopulations in female and male monocultures	5	73	5	27	p > 0.05
Blocking effect on female's lymphocyte subpopulations	15	63	7	25	p > 0.05
Blocking effect on male's lymphocyte subpopulations	23	55	8	24	p > 0.05
Activating effect on female's lymphocyte subpopulations	8	70	11	21	10.05 (OR = 0.23; p < 0.01)
Activating effect on male's lymphocyte subpopulations	23	55	10	22	p > 0.05

TABLE 2. COMPARATIVE ANALYSIS OF THE EFFECTS OF FEMALE AUTOSERUM IN RELATION TO HLA-DR EXPRESSION FOR INDIVIDUAL SUBPOPULATIONS OF FEMALE'S AND MALE'S LYMPHOCYTES IN THE COMPARED GROUPS (DATA ARE PRESENTED IN ABSOLUTE VALUES)

Lymphocyte subpopulations	Case n = 78			Control n = 32			1.1 Chi square (OR; p)	2.2 Chi square (OR; p)	3.3 Chi square (OR; p)
	1. BE	2. NE	3. AE	1. BE	2. NE	3. AE			
HLA $DR^+/CD3^+$, female	34	3	41	14	2	16	p > 0.05	p > 0.05	p > 0.05
HLA $DR^+/CD3^-$, female	49	2	27	13	0	19	4.76 (OR = 2.42; p < 0.05)	p > 0.05	6.01 (OR = 0.37; p < 0.05)
HLA $DR^+/CD45^+$, female	52	0	26	12	0	20	8.31 (OR = 3.25; p < 0.01)	p > 0.05	8.31 (OR = 0.31; p < 0.01)
HLA $DR^+/CD3^+$, male	35	2	41	14	0	18	p > 0.05	p > 0.05	p > 0.05
HLA $DR^+/CD3^-$, male	42	2	34	16	1	15	p > 0.05	p > 0.05	p > 0.05
HLA $DR^+/CD45^+$, male	42	1	35	13	0	19	p > 0.05	p > 0.05	p > 0.05

Note. BE, blocking effect; NE, no effect; AE, activating effect.

creased. If the alleged antibodies to HLA-DR were no contained in the female autoserum, then we suppose no effect on the subpopulations of lymphocytes. On the other hand, further study of the positive effect of female autoserum on the expression of HLA-DR on female's lymphocytes is needed. As can be seen from the presented methodology, the activating effect of female autoserum can be detected during 2 hours of incubation at 37°C. In addition, this effect was dominant for female's lymphocytes in the control group. Short-term changes in the expression of HLA-DR on the membrane of lymphocytes can be attributed to the known functional rearrangements of the receptor apparatus under the influence of new environmental factors resulting to the formation of small receptor clusters. In female autoserum, there can be both dissolved ligands that induce cross-linking of receptors and regulatory molecules that have an activating effect on the short-term reorganization of membrane HLA-DR. It should be additionally noted that the activating effect of female autoserum to HLA-DR expression on subpopulation lymphocytes was positively associated only with lymphocytes of women who underwent all pregnancies and gave birth to two or more healthy children. This autocrine effect by female autoserum factors is aimed, on the one hand, to increase the expression of the HLA-DR molecule on the main subpopulation of $HLA\text{-}DR^+$ lymphocytes, and, on the other, to effective early ontogenesis with inhibition of teratogenesis in the cardiovascular system.

Returning to the problem of positive association of the blocking effect of female autoserum to HLA-DR expression on subpopulations of female's lymphocytes with the formation of sporadic CHD in children without chromosomal diseases, one suggestion can be made. We suggested that the blocking effect is associated with antibodies in the blood serum of women and directed against their own HLA-DR membrane molecules. The fact that the detected blocking activity was maximally manifested on the $HLA\text{-}DR^+/CD3^-$ subpopulation, but not on the $HLA\text{-}DR^+/CD3^+$ subpopulation, only indicates that the last subpopulation was insignificant and the level of HLA-DR expression on T-lymphocytes was initially low. Accepting this position, we can talk that women having children with sporadic CHD without chromosomal diseases have increased autoimmune regulation of HLA-DR restriction of alloantigens, including spousal origin. This immune mechanism may limit the regulatory potential of female T-lymphocytes during pregnancy. As mentioned above, early pregnancy is associated with immune interactions in the mother-embryo system resulting in the balance between compensation and decompensation of local immune inflammation with a predominance of pro-inflammatory interleukins in the blood serum of pregnant women. Female autoantibodies to HLA-DR may be additional humoral factors, leading to a breakdown in local inflammation compensation in the mother-embryo system. The inflammatory process is accompanied by a high level of synthesis of pro-inflammatory cytokines. The action of these molecules will also extend to the embryo with the induction of pyroptosis in the dividing cells of the forming cardiovascular system. It is mechanism that can cause the formation of a malformation in the embryo in the heart and/or in the great vessels.

Thus, the study showed a positive association of the effect of the blocking of female autoserum factors by the expression of HLA-DR on female lymphocytes $HLA\text{-}DR^+/CD3^-$ (OR = 2.42) and HLA $DR^+/CD45^+$ (OR = 3.25) with a risk of sporadic CHD without chromosomal diseases in the next generation.

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