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Changes in Differentiation of Thymic T Cells in the Progeny of Female Mice Subjected to Immune System Stimulation during Early Pregnancy

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Differentiation of T cells was studied in the progeny of female C57Bl/6 mice subjected to immunostimulation by administration of concanavalin A or adoptive transfer of concanavalin A-activated splenocytes. Acceleration of T cell maturation during the prepubertal period and decelerated differentiation during the pubertal and post-pubertal periods were revealed.

Key Words: *T cells; thymus; differentiation; prenatal exposure; concanavalin A*

The relationship between the responses of the maternal immune system during pregnancy and development of the fetal immune system is a topical problem in biology and medicine. Particular attention of embryologists, histologists, immunologists and pediatricians is attracted to the influence of various immunotropic factors on the body during the prenatal period, because they can cause dysfunction of organs and systems in the postnatal ontogeny [3,7,8,10]. The effect of stimulation of the maternal immune system during early pregnancy on the development of the immune system of the fetus is the least studied aspect of this problem.

The aim of this study was analysis of T cell differentiation in the thymus of offspring of female mice subjected to immune system stimulation at early pregnancy terms.

MATERIALS AND METHODS

Experiments were carried out on C57Bl/6 mice. Group 1 males (n=20) were obtained from females receiving single injection of T cell mitogen concanavalin A (Con A, 5 mg/kg; Sigma), activator of lymphocyte proliferation and cytokine secretion, into the orbital sinus on day 7 after fertilization. Pregnancy was confirmed and dated by the presence of sperm in vaginal smear and/or vaginal plug.

Group 2 males (n=20) were obtained from females receiving *in vivo* activated syngeneic splenocytes (AS) on day 7 to prevent direct effects of Con A on the fetus. To this end, intact non-pregnant females were administered with Con A in a dose of 20 mg/kg and 2 h later, splenic cells were isolated, washed 3 times in Hanks solution, brought to a concentration of 50 million cells per 1 ml, and injected into the orbital sinus of females in a volume of 0.2 ml (*i.e.*, 10 million cells per animal) on day 7 of pregnancy. According to calculations, the maximum possible amount of Con A injected with cells was 10 million times lower than the minimum effective dose.

The progeny of intact females and females injected with non-activated syngeneic splenocytes was used as the control. Since there were no significant differences between offspring of these females, they were united into the common control group (n=20).

The mice were sacrificed by zoletil overdose on postnatal day 17 (prepubertal) or in 1.5 months (early puberty) and 2.5 months (post-pubertal period, beginning of active reproductive period) after birth. The thymus was removed and cells were isolated. Subpopulations of thymus lymphocytes were determined by flow cytofluorometry on a Cytomix FC 500 flow cytofluorometer (Beckman Coulter). Fluorochrome-conjugated antibodies (eBioscience Inc.) to mouse

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antigens were used for detection of CD3, CD4, CD8. $CD3^+$ cells were considered T cells. In $CD3^+$ gate, T-helper cells were determined as $CD4^+CD8^-$ cells and cytotoxic T cells were determined as $CD4^-CD8^+$ cells, double positive $CD4^+CD8^+$ cells, and double negative $CD4^-CD8^-$ cells. No less than 10^5 cells were analyzed in the samples.

The data were processed using Statistica 7.0 software (StatSoft Inc.). The central tendency and dispersion of quantitative characters with approximately normal distribution were described by the mean value *M* and standard error of mean *m*. Quantitative variables in independent groups were compared using one-way ANOVA. The differences were considered significant at $p<0.05$.

RESULTS

On postnatal day 17, about 30% of cells in mice of the control group expressed CD3 molecule associated with the T cell receptor (Table 1). $CD4^+CD8^+$ cells constituted 40% of all $CD3^+$ cells and $CD4^-CD8^-$ cells constituted ~2%. The number of differentiated T-helper cells ($CD4^+CD3^+CD8^-$) was comparable to the number of $CD4^+CD8^+$ cells. The fraction of cytotoxic T cells ($CD3^+CD4^-CD8^+$) was ~10% $CD3^+$ cells.

In the progeny of female mice treated with Con A, the number of $CD3^+$ cells among thymus lymphocytes at the age of 17 days was higher by one third than in the control group (Table 1). The fraction of $CD4^+CD8^+$ cells was lower, and the fraction of $CD4^-CD8^-$ cells was higher than in the control group. The fractions of differentiated T-helpers and cytotoxic T lymphocytes among $CD3^+$ cells corresponded to those in the control group.

The number of $CD3^+$ cells in the thymus of AS-treated mice was also higher than in the control, but lower than in the Con A group. The content of $CD4^+CD8^+$ cells did not differ from that in the Con A group and was significantly lower than in the control group. The percentage of $CD4^-CD8^-$ cells was higher than in control, but lower than that in the Con A group. The percentage of T-helper cells did not differ from the control, and the fraction of cytotoxic T lymphocytes surpassed the control values (Table 1).

During pubertal period, a 1.5-fold increase in the percentage of $CD3^+$ thymocytes was revealed in the control group in comparison with the previous term. The fraction of $CD4^+CD8^+$ cells was expectedly reduced by 1.5 times, and the content of $CD4^-CD8^-$ cells was not altered. A significant increase in the content of cytotoxic $CD4^-CD8^+$ T lymphocytes was revealed.

TABLE 1. Changes in Subpopulations of Thymocytes at Different Terms of Postnatal Ontogeny in the Offspring of Female Mice Subjected to Single Immunostimulating Exposure in Early Pregnancy (*M*±*m*)

Thymocytes subpopulation, %		Time of study		
		17 days	1.5 months	2.5 months
$CD3^+$ cells	control	29.47±3.96	43.97±2.25$^+$	43.70±2.61
	Con A	39.13±2.43*	35.32±2.31*	28.71±1.45*
	AS	33.13±3.15	32.90±3.35*	29.48±2.71*
$CD4^+CD8^-$ (% of $CD3^+$)	control	44.20±1.90	49.95±3.32	37.15±1.11$^+$
	Con A	46.95±3.70	45.65±3.45	42.15±2.25*
	AS	47.80±2.85	51.75±3.25	41.20±1.12*$^+$
$CD4^-CD8^+$ (% of $CD3^+$)	control	10.63±0.75	17.87±1.33$^+$	10.60±0.44$^+$
	Con A	11.00±0.33	14.95±0.45*$^+$	11.70±0.45$^+$
	AS	13.50±1.22*	14.60±0.36*	12.50±0.55$^+$
$CD4^+CD8^+$ (% of $CD3^+$)	control	43.26±1.08	30.43±3.23$^+$	50.55±2.16$^+$
	Con A	35.45±2.95*	34.95±3.15	44.10±2.25*$^+$
	AS	34.50±2.75*	29.80±1.98	44.91±2.17*$^+$
$CD4^-CD8^-$ (% of $CD3^+$)	control	2.40±0.17	2.47±0.22	1.20±0.08$^+$
	Con A	6.65±0.45*	4.05±0.35*$^+$	2.15±0.11*$^+$
	AS	4.25±0.65*^{o}	3.90±0.18*	3.10±0.15*^{o}

Note. $p<0.05$ in comparison with *control, ^{o}Con A, $^+$previous investigation term.

At the age of 1.5 months, the number of $CD3^+$ lymphocytes in the thymus in the Con A group did not differ from the previous term and was lower than in the control (Table 1). The fraction of $CD4^+CD8^+$ cells remained unchanged, while the fraction of $CD4^-CD8^-$ cells decreased in comparison with the previous term, but was higher than in the control group. The number of T-helper cells remained unchanged and corresponded to the control values. The fraction of cytotoxic T lymphocytes increased, but did not reach the values of the control group.

The mice of the AS group at the age of 1.5 months did not differ from Con A group mice by the content of $CD3^+$ cells and fractions of $CD4^+CD8^+$ cells, $CD4^-CD8^-$ cells, and differentiated T-helper and cytotoxic T lymphocytes.

At the age of 2.5 months, the number of CD3-expressing cells in control mice was unchanged (Table 1). The content of $CD4^-CD8^-$ cells was reduced, and the fraction of $CD4^+CD8^+$ cells was increased. A decrease in the content of differentiated T-helper and cytotoxic T lymphocytes was noted.

In mice of the Con A group, the content of $CD3^+$ thymocytes slightly decreased after reaching puberty in comparison with the previous term. The percentage of $CD3^+$ cells was by 1.5 times lower than in the control group (Table 1). The percentage of $CD4^-CD8^-$ cells among $CD3^+$ cells decreased in comparison with the previous term, but surpassed the control values. Similar to the control group, an increase in the fraction of $CD4^+CD8^+$ cells was revealed, but it was less pronounced. The content of T-helper cells did not differ from the previous period and surpassed the control values. The fraction of cytotoxic T lymphocytes decreased and approached the corresponding parameter in the control group.

In mice of the AS group in the post-pubertal period the number of $CD3^+$ cells in the thymus did not change and was significantly lower than in the control (Table 1). The content of $CD4^-CD8^-$ cells also remained unchanhed. Their fraction surpassed the control values and values of the reference group. As in the two previous groups, the content of $CD4^+CD8^+$ cells in $CD3^+$ cell population increased in comparison with the previous term and was far below the control. The fractions of T-helper and T-cytotoxic lymphocytes decreased in comparison with the previous term. As in the Con A group, the content of T-helpers surpassed the control values.

It is known that thymus reaches its maximum development during puberty and then undergoes age-related involution [2,4]. In mice, thymus formation begins on embryonic day 9.5 and lymphoid cells can be found in thymus anlage on embryonic day 11.5 [6]. Thus, maternal immune system is stimulated long before the formation of fetal thymus and increased cytokine secretion in response to Con A could not have a direct influence on thymus development and its colonization by precursors of lymphoid cells. Normal development of T cells is initiated in thymus $CD4^-CD8^-$ subpopulations. In thymocytes that had undergone β-selection, expression of $CD4^-$ and $CD8^-$ co-receptors is initiated and later, the cells become single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) [5,9]. In control mice during the pubertal period, differentiation of $CD4^+CD8^+$ thymocytes into single-positive cells was intensified, especially at the expense of reduced CD4 expression resulting in increased differentiation of cytotoxic T lymphocytes. During the post-pubertal period, the increase in the number of T cells was not observed. The rate of differentiation of $CD4^-CD8^-$ cells into $CD4^+CD8^+$ cells increased, and differentiation into single-positive cells was decelerated which attests to the start of age-related thymus involution.

Our results demonstrate that single stimulation of the maternal immune system in early pregnancy prior to embryo's thymus formation causes significant and prolonged changes in thymocyte differentiation. In mice of experimental group, the rate of thymocyte differentiation surpassed the age norm in the prepubertal period. We have previously demonstrated that during this period the spleen of mice of the experimental groups contains a large number of T cells, which confirms acceleration of thymocyte differentiation and migration [1]. In puberty in offspring of females treated with Con A on pregnancy day 7, thymus development was not accompanied by enhanced differentiation of $CD4^+CD8^+$ cells into single-positive cells, but increased early stage of T cell differentiation was observed: CD8 and CD4 were expressed on $CD4^-CD8^-$ cells. In the group of offspring of AS-treated females, transformation of $CD4^+CD8^+$ thymocytes into single-positive was accelerated, the rate of T helper differentiation remained unchanged, and differentiation of cytotoxic T lymphocytes became slower. After sexual maturation the number of T cells in the thymus of experimental animals continued to decline. In the Con A group differentiation of $CD4^-CD8^-$ cells proceeded, but transformation into single-positive cells was slowed down, which was confirmed by increased fraction of $CD4^+CD8^+$ cells. In the AS group the increase of the fraction of $CD4^+CD8^+$ cells was due to deceleration of their differentiation into single-positive cells, especially because CD8 expression was terminated.

Thus, stimulation of the maternal immune system in early pregnancy prior to the formation of fetal thymus can alter thymocyte differentiation at different terms of postnatal ontogenesis of the offspring.

Changes in the rate of thymocyte differentiation are biphasic and opposite to the rate of normal T cell maturation. In the prepubertal period, maturation of T cells, particularly cytotoxic T lymphocytes, was enhanced, and in the pubertal period differentiation processes were decelerated, while normally enhanced thymocyte maturation is observed by the beginning of puberty. Qualitative changes in thymocyte maturation are characterized by activation of early stages of T cell differentiation.

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