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In vivo modulation of the splenocyte yield and composition by female sex steroid hormones

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The study was designed to shed more light on the controversial role of the two main ovarian steroid hormones (i.e. estradiol and progesterone) in shaping the size and phenotypic characteristics of the splenic lymphocyte pool. For this purpose ovariectomized adult rats (OVX) were treated for 14 subsequent days with either estradiol or progesterone (to attain physiological concentrations of the hormones). Afterwards, the splenocyte yield, and overall number of splenocytes bearing TCRαβ receptor, CD4 and CD8 coreceptor were evaluated. Fourteen-day-long ovarian hormone deprivation produced an increase in the splenic weight and splenocyte yield (on the account of a rise in the number of $TCR\alpha\beta^-$ cells), although the number of $TCR\alpha\beta^+$ cells was reduced as a result of a decrease in the size of the $CD4^+$ cell subpopulation. Replacement of either estradiol or progesterone prevented the increase in splenic weight and reduced the splenocyte yield to values significantly lower than that in sham-OVX rats. Both the treatments completely abolished the effect of ovariectomy on the size of $TCR\alpha\beta^-$ cell population, but had differential effects on that of $TCR\alpha\beta^+$ cell population; estradiol did not affect its size, while progesterone caused a reduction on the account of a decrease in the numbers of both $CD4^+$ and $CD8^+$ cells. The results suggest that: a) estradiol and progesterone have similar effects on the size of the splenic B cell population and that replacement of either estradiol or progesterone can prevent the effects of ovariectomy on the size of this population and b) estradiol does not affect while progesterone reduces the size of splenic T cell population. Thus, replacement of none of them is able to compensate the removal of gonads.

1. Introduction

Involution of the thymus, the site of T cell differentiation, has long been recognized to occur during pregnancy in number of species including rat, mouse and human [1–4]. It has been assumed that these changes in the thymus result in reduced or altered output of the mature T cells into the periphery. The pregnancy-induced involution of this organ may be mimicked by administration of ovarian steroid hormones (i.e. estrogens and progesterone), although the relative contributions of the two hormones are still controversial [5–8]. Moreover, it has been reported that during pregnancy, similarly to the thymus, the bone marrow, the site of B lymphocyte development, also undergoes an involution process leading to a specific block in B cell development. This effect of pregnancy can be replicated in non-pregnant females by implantation of estradiol and progesterone containing pellets to produce levels of hormones similar to those achieved during pregnancy [9–11]. The physiological significance of pregnancy-induced involution of the thymus and bone marrow has not been fully understood yet, but it has been supposed that it represents an important component of maternal immunoregulation essential for normal fertility.

This study was designed to enlighten the still controversially discussed role of the two main ovarian steroid hormones (i.e. estradiol and progesterone) in regulation of the output of mature lymphocytes from the sites of their differentiation, and consequently phenotypic and functional shaping of the splenic lymphocyte pool. For this purpose the splenocyte yield and overall numbers of splenocytes bearing TCRαβ receptor, CD4 and CD8 coreceptor molecules were determined in ovariectomized adult rats (OVX) treated daily for 14 subsequent days with vehicle alone, estradiol or progesterone (to attain physiological concentrations of the hormones).

2. Investigations and results

2.1. Serum concentrations of estradiol and progesterone

Efficiency of the steroid hormone substitution treatment was evaluated by determination of the serum concentration of estradiol and progesterone, respectively. The serum estradiol concentration in OVX + ET rats (640.0 ± 15.0 pmol/ml, $n = 6$) did not significantly ($p > 0.05$; t-test, d.f.10) differ from that of sham-OVX rats (622.4 ± 25.0 pmol/ml, $n = 6$). Similarly, there was no significant ($p > 0.05$; t-test; d.f.10) difference between the serum progesterone concentration of OVX + PT rats (97.6 ± 5.2 nmol/l, $n = 6$) and sham-OVX controls (116.1 ± 12.2 nmol/l, $n = 6$).

2.2. Splenic weight and splenocyte yield

Since neither spleen weight nor splenocyte yield significantly ($p > 0.05$, t-test, d.f.10) differed between OVX and OVX-vehicle-treated rats the data from these two groups were pooled for analysis and presentation.

Ovariectomy produced a significant ($p < 0.01$) increase in the splenic weight. Both estradiol and progesterone admin-

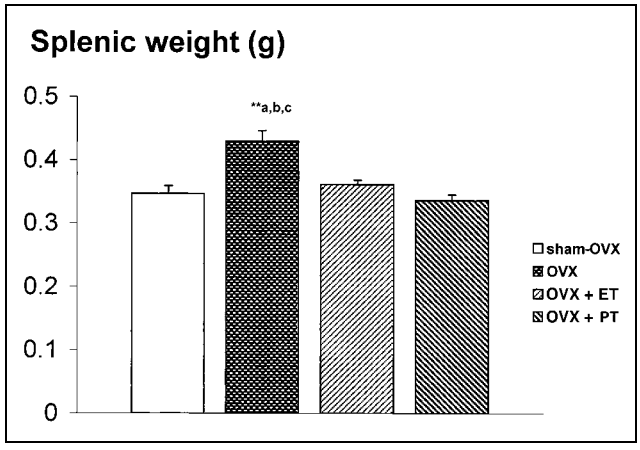

Fig. 1.: The splenic weight in adult rats sham-ovariectomized (sham-OVX); OVX, OVX and treated for 14 consecutive days with estradiol (OVX + ET) and progesterone (OVX + PT).
The results are expressed as mean ± SEM (n = 5–10)
** $p < 0.01$; [a] sham-OVX vs. OVX; [b] OVX vs. OVX + ET; [c] OVX vs. OVX + PT

Table: Splenocyte yield and absolute numbers of splenocytes expressing TCRαβ, CD4 and CD8 molecules in adult rats sham-ovariectomized (sham-OVX), OVX, OVX and treated for 14 consecutive days with estradiol (OVX + ET) or progesterone (OVX + PT)

	Splenocytes				
Groups of rats	Yield ($\pm 10^7$)	TCRαβ$^-$ ($\pm 10^7$)	TCRαβ$^+$($\pm 10^7$)	CD4$^+$ ($\pm 10^7$)	CD8$^+$ ($\pm 10^7$)
sham-OVX	25.15 ± 0.32	9.55 ± 0.81	15.48 ± 0.59	9.92 ± 0.55	5.56 ± 0.27
OVX	27.15 ± 0.06*a,**b, c	15.75 ± 0.52*a, b, c	11.16 ± 0.54**a, c	5.79 ± 0.28**a, c	5.50 ± 0.26**c
OVX + ET	19.35 ± 1.09**a	10.26 ± 0.98	9.08 ± 0.52**a	5.61 ± 0.52**a	4.79 ± 0.30
OVX + PT	18.38 ± 1.61**a	9.92 ± 1.02	8.10 ± 0.48**a	4.73 ± 0.12**a	3.38 ± 0.56**a

The results are expressed as mean ± SEM (n= 5–10)
* $p < 0.05$; ** $p < 0.01$; [a]sham-OVX vs. OVX, OVX + ET, OVX + PT; [b] OVX vs. OVX + ET; [c] OVX vs. OVX + PT

istrations prevented the increase in splenic weight induced by ovariectomy. Thus, the value of this parameter neither in OVX + ET nor in OVX + PT rats was distinguishable from that in sham-OVX rats (Fig. 1).
Ovariectomy also evoked small but significant ($p < 0.05$) increase in splenocyte yield, while the replacement of either estradiol or progesterone, not only prevented this effect of ovariectomy, but evoked a reduction in splenocyte yields to value which were significantly ($p < 0.01$) less than in sham-OVX rats (Table).

2.3. Analysis of the expression of TCRαβ, CD4 and CD8 on splenocytes

There was no significant differences between OVX and OVX-vehicle-treated rats in expression of any of the analyzed molecules and the data from these two groups were therefore pooled.
Ovariectomy significantly ($p < 0.01$) increased the absolute number of TCRαβ$^-$ splenocytes, accompanied by an opposite effect on TCRαβ$^+$ cells. Thus the TCRαβ$^-$/TCRαβ$^+$ cell ratio was significantly increased in rats subjected to 14-day-long ovarian hormone deprivation (0.62 ± 0.06 in sham-OVX vs. 1.41 ± 0.09 in OVX rats; $p < 0.01$). Administration of either estradiol or progesterone in OVX rats significantly ($p < 0.01$) decreased the absolute number of TCRαβ$^-$ splenocytes. The number of these cells in neither OVX + ET nor in OVX + PT rats significantly differed from that in sham-OVX. However, since ET had no effect on the absolute number of TCRαβ$^+$ cells, while PT significantly ($p < 0.01$) reduced their absolute number (Table), the TCRαβ$^-$/TCRαβ$^+$ cell ratios were significantly decreased in both OVX + ET and OVP + PT rats (1.41 ± 0.09 in OVX vs. 1.12 ± 0.03 in OVX + ET and 1.22 ± 0.04 in OVX + PT rats; $p < 0.01$).
Ovariectomy significantly ($p < 0.01$) reduced the absolute number of CD4$^+$ cells, but did not affect significantly the absolute number of CD8$^+$ cells (Table), the CD4$^+$/CD8$^+$ cell ratio was significantly reduced in OVX rats compared with sham-operated controls (1.78 ± 0.04 in sham-OVX vs. 1.05 ± 0.03 in OVX rats; $p < 0.01$). Estradiol replacement significantly influenced the absolute number of neither CD4$^+$ nor CD8$^+$ splenocytes and therefore the CD4$^+$/CD8$^+$ cell ratio remained unaltered (1.05 ± 0.03 in OVX vs. 1.27 ± 0.06 in OVX + ET rats; $p > 0.05$). On the other side, progesterone injections produced a significant reduction in the absolute number of both CD4$^+$ ($p < 0.05$) and CD8$^+$ splenocytes ($p < 0.01$) (Table) causing an increase in the CD4$^+$/CD8$^+$ cell ratio (1.05 ± 0.03 in OVX vs. 1.40 ± 0.04 in OVX + PT rats; $p < 0.05$).

3. Discussion

The results showed that: a) 14-day-long ovarian hormone deprivation produced an increase in the splenic weight and splenocyte yield (as a result of a rise in the absolute number of TCRαβ$^-$ cells), although the absolute number of TCRαβ$^+$ cells were reduced reflecting a decrease in the size of CD4$^+$ cell population; b) replacement of either estradiol or progesterone prevented the increase in the splenic weight and caused a reduction in the splenocyte yield (to values significantly lower than in sham-OVX rats) completely abrogating the effect of ovariectomy on the size of TCRαβ$^-$ cell population and either non affecting (estradiol) or diminishing the size of TCRαβ$^+$ cell population on the account of a decrease in the number of both CD4$^+$ and CD8$^+$ cells (progesterone).
The hereby reported enlargement of the spleen in rats subjected to 14-day-long ovarian hormone deprivation is in agreement with some previous reports [12]. The increase in the splenocyte yield in these animals, most likely, reflected a marked expansion of the TCRαβ$^-$ cell compartment accordingly to the observations that ovariectomy augments B lymphopoiesis and that estrogens are negative regulator of B cell development *in vivo* and *in vitro* [9, 13, 14]. The major effects of estrogens in regulation of B lymphopoiesis appear to require the presence of bone marrow stromal cell-derived factors [15, 16] and to occur at the pre-B cell stage, thus that the number of IL-7 responsive cells is markedly decreased in estrogen-treated animals [11]. In the same line is the present finding that administration of estradiol completely abolished the effects of ovariectomy on the number of TCRαβ$^-$ cells in the spleen. However, since progesterone treatment had a similar effect to that evoked by estradiol, it seems that not only estrogens, but possibly progesterone is a negative regulator of B lymphopoiesis, as well. In favor of this thesis are data showing that cells from the primary site of B cell maturation contain progesterone receptors [17]. On the other hand, since data on the presence of the estrogen receptors on the peripheral B cells are controversial [18, 19], the modulation of size of B cell splenic population via estrogen action at the spleen level cannot be excluded.
Unexpected decrease in size of the TCRαβ$^+$ splenic cell population most likely reflected a net effect of the lack of ovarian hormone action on the intrathymic T cell maturation, on one side, and on the homing, apoptosis and proliferative capacity of the mature T cells, on the other side. Namely, it has been shown that ovariectomy significantly increases the absolute numbers of both the most mature single positive CD4$^+$ and CD8$^+$ thymocytes [20]. However, it has also been demonstrated that the ovarian hormone deprivation decreases the splenic homing of injected

T cells [21]. In accordance with the latter finding are data that both estradiol and progesterone have significant role in regulation of the expression of endothelial adhesion molecules [22]. Moreover, it has been shown that estradiol significantly increases the blastogenic response of spleen cells to T lymphocyte mitogens [23, 24]. Finally, recently reported data showing that estradiol prevents Fas-dependant apoptosis in $CD4^+$ T cells, by altering Bcl-2 expression [25], can be related to a significant reduction in number of $CD4^+$ splenocytes which, most likely, resulted in a drop of overall number of $TCR\alpha\beta^+$ cells and caused a shift in the $CD4^+/CD8^+$ cell ratio toward $CD8^+$ cells in the OVX rats.

It has already been shown that estradiol replacement in OVX rats significantly decreases the absolute number of the most mature single positive $CD4^+$ and $CD8^+$ intrathymic T cells [20]. However, since the number of $TCR\alpha\beta^+$ cells in spleen from OVX + ET rats remained unaltered, it seems that in these rats the decrease in output of mature T cell from the thymus was fully compensated by the effects of estradiol on the spleen homing of T cells [21] and on splenic T cells sensitivity to proliferative and apoptotic stimuli [23–25].

The drop in the number of $TCR\alpha\beta^+$ cells in spleen of OVX + PT vs. OVX rats can be associated with the findings showing that PT in OVX rats did not affect the absolute number of the most mature single positive $CD4^+$ and $CD8^+$ intrathymic T cells [20], on one side, and with those indicating that splenocytes cultured in the presence of progesterone release a cellular factor that inhibits proliferation of spleen cells [26], on the other side.

Finally, although both estradiol and progesterone reduced the splenocyte yields to a value significantly lower than in sham-OVX rats, the weights of their spleens were reduced thus to be indistinguishable from that in sham-operated controls, that clearly suggest that in both OVX + ET and OVX + PT rats the red pulp compartment was enlarged. In support of this thesis it can be added that both estrogen and progesterone markedly increase the red pulp volume [27].

In conclusion, estradiol and progesterone shape the size and phenotypic characteristics of the splenic lymphoid cell pool acting on both the B and T cell lymphopoiesis and on homing, apoptosis and proliferative capacity of the mature lymphocytes. These two hormones have: a) similar effects on the size of the splenic B cell population and administration of only one of them can prevent the increase in the size of this population evoked by ovariectomy and b) differential effects on the size and phenotypic characteristics of splenic T cell population, thus that replacement of none of them alone is able to compensate the removal of gonads. Hence, it seems obvious that full establishment of the size of splenocyte population, as well as its phenotypic and consecutively functional characteristics in gonadal hormone deficient rats requires replacement of both the ovarian hormones.

4. Experimental

4.1. Animals

Female AO rats old 90 day at the beginning of experiment were used in the study hereby presented. The rats were bilaterally ovariectomized (OVX) or sham-operated (sham-OVX) under sodium pentobarbitone anesthesia (Nembutal, Serva, 40 mg/kg b.w.). The OVX rats were divided into two groups: rats from one group were not further treated, while rats from the other group were subjected randomly to one of the following experimental conditions: a) estradiol treatment (OVX + ET), b) progesterone treatment (OVX + PT) or c) vehicle treatment (vegetable oil administration, OVX + OT). Thus for 14 consecutive days, beginning from the first day after ovariectomy, the animals were treated with s.c. injections of: a) estradiol (Estradiol, ICN, Galenika, Yugoslavia; 2.5 μg/100 g/day), b) progesterone (Progesteron ICN, Galenika, Yugoslavia; 1 mg/100 g/day) and c) vegetable oil (50 μl/100 g/day), respectively. Sham-operated animals were untreated but subjected to daily vaginal smear analyses. Due to practical constraints the sham-operated animals were not all at the same stage of the cycle at autopsy, but the study included only those animals which showed two consecutive regular 4-day estrous cycles. Each group consisted of at least 5 rats. Fourteen days after ovariectomy, or sham-surgery, all animals were killed by decapitation. Their spleens were removed, carefully dissected and weighed.

4.2. Flow cytometry analysis

For flow cytometry analysis (FCA) the splenocyte suspensions were prepared by grinding the splenic tissue between the frosted ends of microscope slides and passing the resultant suspension through a fine nylon mesh. The single-cell suspension so obtained was washed once in ice-cold phosphate-buffered saline (PBS, pH 7.3) containing 5% fetal calf serum (Gibco, Grand Island, NY) and 0.01% sodium azide (PS medium) and then treated with 2 ml of erythrocyte lysing solution (0.83% ammonium chloride in 0.17 M TRIS-solution), pH 7.2. After 10 min suspensions were washed twice in ice-cold PBS, and the cell concentration was adjusted to 1×10^7/ml. The viability of such cell preparations, as determined by Trypan blue exclusion, was routinely greater than 95%.

Aliquots of 1×10^6 splenocytes in 100 μl PS medium were dispensed into conical microcentrifuge tubes, centrifuged to yield a pellet, and the supernatant fluid was decanted. The cells were incubated for 30 min on ice with one of the following anti-rat monoclonal antibodies (mAb): anti-CD4 (W3/25, Serotec, Oxford, UK); anti-CD8 (MRC 0X-8, Serotec) and anti-TCRαβ (R73, Serotec), with isotypic controls or with the same amount of PS as first step reagents. Following the incubation, the splenocytes were washed three times in PS medium and then incubated for another 30 min on ice with the second step reagent (anti-mouse IgG-FITC antibody, INEP, Zemun, Yugoslavia) and again washed three times in the same medium.

After labeling, the cells were fixed in 0.5 ml 1% paraformaldehyde and kept at 4 °C in the dark until analysis. All samples were analyzed on the same day on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Dead cells and debris were excluded from analysis by selective gating based on anterior and right angle scatter. 5×10^3 flow cytometric events were analyzed. The analysis was carried out with respect to appropriate isotypic and fluorochrome-matched controls, with Consort 30 software (Becton Dickinson).

4.3. Serum concentration of sex steroid hormones

After decapitation of animals, trunk blood was collected, serum separated and stored at −20 °C until radioimmunoassay (RIA) was performed. Estradiol and progesterone concentrations were measured using the ESTRCTRIA kit (CIS bio international, Gif-sur-Yvette, France) and the RIA Progesteron kit (INEP Dijagnostika, Zemun, Yugoslavia), respectively. The RIA procedures were carried out according to the guidelines provided by manufacturers.

4.4. Statistical analysis

All data are expressed as mean ± S.E.M. Data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's test for comparison of different mean values.

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Received July 26, 2000
Accepted August 25, 2000

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