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# The influence of 17β-estradiol benzoate on weight and cellularity of rat lymphoid tissue and peripheral blood

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## *INTRODUCTION*

In the last few years research on sex steroid-induced alterations has become increasingly important in basic and applied reproductive biology and medicine. Furthermore, new contraceptive methods and estrogen therapies have been introduced (*Hosie et al.* 1989, *Takano-Yamamoto & Rodan* 1990). Thus, pharmacology and toxicology of estrogens have been established as important fields of research. For various reasons ovariectomized female rats are the animal model of choice for these investigations. Animal models with ovariectomized female rats are also very attractive for studying $E_2$-mediated variations of mineral and energy metabolism and endocrine modifications.

In addition to metabolic and endocrine changes, a few examinations indicate that estrogens have profound influences on lymphoid tissue mass and structure (*Nelson et al.* 1967, *Reilly et al.* 1967, *Sobhon & Jirasattham* 1974) and it is not yet clear how important any of these modifications are in physiological, pharmacological and toxicological research of estrogens. The main effect of $E_2$ administration in various rodents is marked by thymic and occasionally peripheral lymphoid involution (*Nelson et al.* 1967, *Sobhon & Jirasattham* 1974, *Kalland et al.* 1978). It is on the other side surprising how little information is available about the distribution of lymphocyte populations in long-term estradiol-treated, ovariectomized female rats. Therefore, we investigated 17β-estradiol benzoate-treated, ovariectomized female Lewis rats with special reference to lymphoid tissue mass and cellularity, peripheral white blood cell parameters and the distribution of T and B lymphocytes of various lymphoid organs. For comparison, age-matched vehicle-injected, ovariectomized animals were included in the investigation.

## *MATERIAL AND METHODS*

### *Animals*

A total of 60 adult female Lewis strain rats were employed in the present study. The animals were obtained from Zentralinstitut für Versuchstierzucht (Hannover, FRG) and housed in groups of three rats. The animals were reared in a conventional environment in air conditioned rooms, 20±2°C, with a photoperiod of 12 hours light, 12 hours dark (light on at 07:00 h), with free access to Ssniff rat chow (Ssniff, Soest, FRG) and water. During the experiment, each rat was housed individually in a polypropylene cage.

### *Experimental protocol*

Female inbred Lewis rats (LEW/Han) were distributed among 4 examination groups with 15 animals per group: ovariectomy (OV; removal of both ovaries) and injection of corn oil (OV/corn oil); ovariectomy and injection of 1 μg 17β-estradiol 3-benzoate/kg body weight (OV/1μg $E_2$); ovariectomy and injection of 5 μg $E_2$/kg BW (OV/5μg $E_2$); and ovariectomy and application of 50 μg $E_2$/kg BW (OV/50μg $E_2$). Surgeries were performed on adult rats, 63±3 days of age. Under xylazin/ketamine anesthesia the abdomen was opened by ventral incision in the *Linea alba* and ovaries were removed.

Applications of corn oil *resp.* $E_2$ dissolved in corn oil were performed by intramuscular injections on a 4-day cycle starting 17 days after surgical procedures. In the vehicle-treated, ovariectomized group, corn oil (Sigma Chemical Company, St. Louis, USA) was administered at a dosage of 0.1 ml/kg BW until the animals were sacrificed. $E_2$, dissolved in corn oil, was injected for 72 days at doses of 1, 5, or 50 µg/kg BW to ovariectomized groups. The application profile of corn oil *resp.* $E_2$ in corn oil every fourth day is similar to the endogenous $E_2$ surge of the estrous cycle (*Butcher et al.* 1974).

Blood was taken in the morning between 8:00 and 10:00 h from the retroorbital plexus under light ether anesthesia to determine circulating white blood cell counts. The animals were bled at the age of 154 days when they were sacrificed by decapitation. Blood was collected within 1 min after the onset of handling the animal.

*Peparation of organ lymphocytes*

Thymus, spleen and Lnn. iliaci mediales (LLN) were excised immediately after sacrificing the animals and weighed after removing the adhering tissue and para-thymic lymph nodes. Cell suspensions were prepared by pressing the organs through sterile gauze in ice-cold Hanks' balanced salt solution (HBSS; Serva, Heidelberg, FRG). Unless otherwise stated, the medium used for immunofluorescence (IF) staining was HBSS containing 5 % bovine serum albumin (BSA; Serva, Heidelberg, FRG). Cell counts of lymphoid organs were quantified with a Coulter counter. Cell viability was determined by staining the cells by trypan blue exclusion (0.05 % in saline). Phagocytic mononuclear cells were identified by the latex bead ingestion technique as described by *Senogles et al.* (1979). To determine the distribution of pan T, T helper, T cytotoxic/suppressor and B lymphocytes, aliquots of the cell suspensions were incubated with latex beads and antibodies specific to surface markers of lymphocytes. The direct membrane IF technique of *Winchester & Ross* (1976) was used to identify B lymphocytes. Briefly, thymus, spleen and lymph node lymphocytes were washed three times in HBSS-BSA and aliquots of $5 \times 10^6$ cells were pelleted in glass tubes. Fluorescein isothiocyanate (FITC)-conjugated affini pure goat anti-rat IgG, F(ab')2 fragment specific (Jackson Immunoresearch Laboratories, Inc., Baltimore, USA) was diluted 1:20 in HBSS-BSA, and 0,1 ml was added to the cell pellet. After 30 min incubation at 4°C, the cells were washed in cold HBSS-BSA three times and examined under a fluorescence microscope. At 630× magnification at least 200 cells were scored. Only cells showing discrete surface patches were scored as being B lymphocytes and any cells showing a dull homogeneous staining pattern were considered dead and rejected from all tabulations (*Clancy & Mauser* 1981). Pan T lymphocyte fraction, T helper (CD4) and T suppressor/cytotoxic (CD8) cells were identified by indirect IF technique employing murine monoclonal anti-rat pan T lmphocyte antibody (W3/13 HLK; Sera-lab, Sussex, UK), anti-rat T helper cell (W3/25; CD4 equivalent; Sera-lab, Sussex, UK) and anti-rat non-helper subset T cell antibody (OX8; Lyt2/Lyt3 or CD8 equivalent; Sera-lab, Sussex, UK). Briefly, $5 \times 10^6$ cells were prepared as described above and incubated with 0.1 ml of a 1:100 dilution of murine monoclonal anti-rat antibodys W3/13 HKL, W3/25 and OX8, respectively, for 60 min at 4°C. Then cells were washed three times in HBSS-BSA and 0.1 ml of fluorescein conjugated affini pure rat anti-mouse Igg (Jackson Immunoresearch Laboratories, Inc., Baltimore, USA), diluted 1:40, was added to the cell pellet. Cells were incubated for 30 min at 4°C in the dark, washed three times, and examined under a fluorescence microscope. At least 200 cells were counted for each sample as above.

*Preparation of blood cells*
Aliquots of blood samples from day 154 were used for preparation of white blood cell numbers and distribution of T and B lymphocytes. Total leukocyte counts were quantified with a Coulter counter. Smears prepared from blood for differential leukocyte analyses were stained with M+D Diff-Quick stain (Merz and Dade AG, Düdingen, Switzerland). The total numbers based on total and differential cell counts were calculated. To determine the distribution of pan T and B lymphocytes in peripheral blood, circulating mononuclear cells were separated from heparinized blood on a Ficoll-Paque gradient (Sigma Chemical Company, St. Louis, Missouri) and washed three times in hank's balanced salt solution (HBSS; Serva, Heidelberg, FRG). Phagocytic mononuclear cells were identified by the latex bead ingestion technique as described above. The direct membrane IF technique was used to identify B lymphocytes and T lymphocytes were determined by indirect IF technique as described previously.

*Statistics*
Statistical analysis of data was performed by Kruskal/Wallis test and Duncan's multiple range test (*SAS Institute* 1985). The results were considered statistically significant if $p < 0.05$.

## *RESULTS*

*Lymphoid organs*
As shown in Table 1, $E_2$ administration decreased body weight moderately to significantly at 5 *resp.* 50 µg $E_2$/kg BW. Absolute and relative thymus weight was significantly reduced at 5 *resp.* 50 µg $E_2$/kg BW, whereas 1 µg $E_2$/kg BW failed to induce significant changes. In absolute and relative spleen weight, a significant decrease was noted between vehicle-injected, ovariectomized females and $E_2$-treated (50 µg $E_2$/kg BW), ovariectomized animals. For the absolute and relative LLN weight, no significant changes were observed after $E_2$ application.

Cellularity of thymus and spleen in vehicle- and $E_2$-administered, ovariectomized rats followed the same pattern as lymphoid organ weights (Table 2). Significant differences observed in the results obtained from thymus and spleen cellularity concerned corn oil-injected and $E_2$-treated (50 µg/kg WW), ovariectomized animals while in LLN no significant differences have been observed.
For relative numbers of latex bead phagocytizing mononuclear cells, pan T lymphocyte fraction, helper T lymphocytes (CD 4), cytotoxic/suppressor T cells (CD 8) and B lymphocytes in spleen and Lnn. iliaci mediales as well as latex bead phagocutizing mononuclear cells, pan T cells, helper T lymphocytes (CD 4) and cytotoxic/suppressor T cells (CD 8) in thymus no significant modifications were noted in $E_2$-injected animals (data not shown).

*Peripheral white blood cell counts*
For peripheral white blood cells, the absolute numbers of leukocytes, lymphocytes and monocytes revealed a moderate decrease due to increasing $E_2$ doses while band neutrophils showed a moderate increase (Table 3). As shown in Table 4, the percentage of pan T cells and B lymphocytes in the peripheral blood revealed no obvious differences between the groups.

## *DISCUSSION*

In ovariectomized female Lewis rats used in the above investigation, application of $E_2$ modified weight and cellularity of lymphoid organs. Exogenous $E_2$ revealed suppressive effects on thymus and spleen mass and cellularity. Thymic weight loss by $E_2$ application was expected since it has been observed previously (*Sobhon & Jirasattham* 1974). The action of $E_2$ on the thymus seems to be mediated via estrogen receptors that have been demonstrated in rat thymic medullary tissue (*Grossman et al.* 1979, *Stimson et al.* 1980). In contrast to changes in thymic weight and cellularity the distribution of surface markers of lymphocytes was not signifi-

*Table 1.* Body, thymus, spleen and lumbar lymph node (LLN) weight in corn oil- and 17β-estradiol benzoate ($E_2$)-injected, ovariectomized (OV) female Lewis rats.

| | | OV/corn oil | OV/1μg $E_2$ | OV/5μg $E_2$ | OV/50μg $E_2$ |
|---|---|---|---|---|---|
| Body weight (g) | | 239±17 | 242±22 | 226±10 | 229±12 |
| Thymus weight | abs. | 288±34 | 317±53 | 239±17 | 160±14 |
| | rel. | 1.21±0.16 | 1.35±0.21 | 1.06±0.10 | 0.71±0.06 |
| Spleen weight | abs. | 402±45 | 424±37 | 369±59 | 321±31 |
| | rel. | 1.72±0.22 | 1.74±0.23 | 1.64±0.25 | 1.41±0.09 |
| LLN weight | abs. | 35.4±12.9 | 39.1±14.1 | 38.7±10.9 | 30.3±8.2 |
| | rel. | 0.13±0.04 | 0.16±0.06 | 0.17±0.05 | 0.13±0.04 |

Results are given as group mean ± SD. There were 15 animals in each group. Ovariectomized female Lewis rats were treated with corn oil *resp.* 1, 5 or 50 μg $E_2$ for 72 days. Absolute organ weights (abs.) are expressed in mg and relative lymphatic organ weights (rel.) as percentage of organ weight in mg/g body weight.
* = significant difference between two groups of animals using Duncan's multiple range test: $p < 0.05$.

*Table 2.* Cellularity of thymus, spleen and LLN in vehicle-injected (corn oil) or $E_2$-treated (1, 5 *resp.* 50 μg $E_2$/kg BW), ovariectomized (OV) female Lewis rats.

| | OV/corn oil | OV/1μg $E_2$ | OV/5μg $E_2$ | OV/50μg $E_2$ |
|---|---|---|---|---|
| Thymus | | | | |
| nucleated cells x $10^8$/organ | 7.7±1.6 | 8.5±1.6 | 6.2±0.8 | 3.6±0.5 |
| Spleen | | | | |
| nucleated cells x $10^8$/organ | 5.1±0.7 | 5.1±0.7 | 4.5±0.8 | 3.7±0.5 |
| LLN | | | | |
| nucleated cells x $10^7$/organ | 3.4±1.1 | 3.3±1.3 | 3.6±1.1 | 2.8±0.8 |

Explanations as in Table 1.

*Table 3.* Absolute leukocyte parameters in the peripheral blood of corn oil- and $E_2$-injected, ovariectomized (OV) female Lewis rats.

| | OV/corn oil | OV/1µg $E_2$ | OV/5µg $E_2$ | OV/50µg $E_2$ |
|---|---|---|---|---|
| Total white cell count (× $10^9$/l) | 8.5±2.3 | 8.2±2.6 | 6.9±2.0 | 6.9±1.9 |
| Absolute lymphocyte count (× $10^9$/l) | 7.2±2.0 | 6.8±1.8 | 5.7±1.6 | 5.7±1.8 |
| Absolute segmented neutrophil count (× $10^9$/l) | 0.9±0.2 | 1.1±0.4 | 0.8±0.4 | 0.9±0.3 |
| Absolute band neutrophil count (× $10^8$/l) | 2.0±1.7 | 2.8±1.6 | 2.9±2.4 | 2.6±2.3 |
| Absolute eosinophil count (× $10^8$/l) | 0.3±0.2 | 0.5±0.4 | 0.3±0.2 | 0.3±0.3 |
| Absolute monocyte count (× $10^8$/l) | 1.5±1.0 | 1.2±1.0 | 1.1±0.9 | 0.9±0.7 |

Explanations as in Table 1. Basophils were not observed. Results are given as mean ± SD.
There were 15 animals in each experimental group.
Significant differences between the groups were not observed.

cantly influenced by $E_2$ administration, indicating that there was a proportional reduction of all examined lymphocyte populations.

Estrogens cause thymic involution (*Dougherty* 1952, *Sobhon & Jirasattham* 1974), but their effects on other lymphoid organs were minimal and closely related to the species studied in previous investigations (*Dougherty* 1952, *Reilly et al.* 1967, *Sobhon & Jirasattham* 1974). However, in the present investigation a moderate to significant decrease of spleen mass and cellularity was observed, but the reduction showed a lesser extend than in the thymus. Splenic mass reduction by $E_2$ administration has also been reported (*Sobhon & Jirasattham* 1974) and was thought to be due to the direct influence of $E_2$ on the spleen since estrogen receptors were found in the cytosol of the mouse spleen (*Detlefsen et al.* 1977). In agreement with observations in the thymus, the distribution of lymphocyte surface markers showed no significant differences between the groups.

In agreement with our findings *Clancy & Mauser* (1981) also found no significant differences in relative T and B lymphocyte

*Table 4.* Percentage of T and B lymphocyte populations and phagocytizing mononuclear cells in the peripheral blood of long-term $E_2$-treated, ovariectomized (OV) female Lewis rats.

| | OV/corn oil | OV/1µg $E_2$ | OV/5µg $E_2$ | OV/50µg $E_2$ |
|---|---|---|---|---|
| Latex bead phagocytizing mononuclear cells | 21±12 | 20±10 | 18±6 | 16±8 |
| Pan T lymphocytes (W3/13 HLK) | 77±8 | 79±6 | 75±7 | 82±11 |
| B lymphocytes | 14±5 | 13±5 | 12±6 | 13±5 |

Explanations as in Table 1. Values are expressed as groups mean ± S.D. of positive cells by direct or indirect IF assay.
Significant differences were not observed.

numbers of thymus, spleen and lymph nodes detected in male versus female rats. In contrast to the above results *Colombo et al.* (1988) found variations of the T/B lymphocyte relationship in the uterus draining lymph nodes during the estrous cycle.

$E_2$ has been shown in previous studies to have suppressive actions on peripheral white blood cell counts and circulating lymphocyte counts (*Reilly et al.* 1967, *Sobhon & Jirasattham* 1974). In the above study, however, the total white cell counts and lymphocyte numbers were only moderately reduced in $E_2$-treated compared to vehicle-injected control groups while absolute counts of band neutrophils were moderately increased. Thus, the present data tend to results of *Sobhon & Jirasattham* (1974) that $E_2$ suppresses the number of circulating lymphocytes while stimulating more neutrophil formation and release from bone marrow. Results of *Byron* (1971) may help to explain the moderate decrease of circulating leukocyte and lymphocyte counts. Steroid hormones influence mammalian hematopoiesis by a variety of mechanisms. Steroid hormones have been shown to affect marrow growth directly by acting on several classes of cells, including uncommitted and committed hematopoietic stem cells (*Byron* 1971). Finally, similar to findings in lymphoid organs, the distribution of T and B lymphocytes in the peripheral blood was not affected by $E_2$ administration in the present study.

In conclusion, long-term administration of $E_2$ affects thymus and spleen weight and cellularity while Lnn. iliaci mediales, peripheral blood parameters and the distribution of T and B lymphocyte populations were in a lesser extend influenced.

*Summary*

The effects of 17β-estradiol benzoate ($E_2$) upon weight and cellularity of thymus, spleen and Ln. iliaci mediales (LLN), peripheral white blood cell parameters as well as T and B lymphocyte distribution were studied in vehicle- and $E_2$-injected, ovariectomized female Lewis rats. The effects seen by $E_2$ administration were a decrease in weight and cellularity of thymus and spleen while LLN weight and cell counts showed no significant differences between the groups. Circulating leukocyte counts exhibited a moderate decrease in $E_2$-injected animals. On the other side the distribution of pan T, T helper, T cytotoxic/suppressor and B lymphocytes in the examined organs and peripheral blood was not affected by 17β-estradiol benzoate treatment. In conclusion, the present results suggest that 17β-estradiol benzoate reduces weight and cellularity of thymus and spleen while Lnn. iliaci mediales, peripheral white blood cell counts and the distribution of lymphocyte surface markers of the examined lymphoid tissue were only weakly to moderately influenced by $E_2$ administration to ovariectomized female Lewis rats.

*Sammendrag*

Effekten af 17-β-østradiol-benzoat ($E_2$) på vægten og cellulariteten af de tre organer thymus, milt og Ln. iliaci med. (LLN), perifere leucocyt-parametre samt T- og B-celle-fordeling studeredes i vehikel- og $E_2$-injicerede, ovariektomerede Lewis rotter. Den observerede effekt af $E_2$-behandling var en reduktion i vægten og cellulariteten af thymus og milt, mens vægt og cellularitet af LLN ikke var signifikant forskellig grupperne imellem. Det perifere leucocyt-tal var en smule forøget i $E_2$-injicerede dyr. Imidlertid var fordelingen af pan-T-, T-hjælpe- og T-cytotoksisk/suppressor- og B-lymphocyter i de undersøgte organer ikke påvirket af behandlingen med 17-β-østradiol-benzoat. Det konkluderes, at disse resultater indikerer, at 17-β-østradiol-benzoat er i stand til at reducere vægt og cellularitet af thymus og milt, mens Lnn. iliaci med., perifert leucocyt-tal samt fordelingen af lymfocytære overflade-markører i det undersøgte lymfoide væv kun i ringe til moderat grad påvirkes af indgift af $E_2$ til ovariektomerede Lewis hun-rotter.

*Yhteenveto / P. Pelkonen*

Lewis-naarasrotissa, joilta oli poistettu munasarjat, tutkitiin 17-β-estradiolibentsoaatin ($E_2$) vaikutuksia kateenkorvan, pernan ja lantion alueen imusolmukkeiden painoon ja solumääriin, perifeerisiin valkosolumuuttujiin ja T- ja B-tyypin imusolujen jakautumaan. $E_2$-käsitellyissä eläimissä kateenkorvan ja pernan solumäärät ja paino laskivat, mutta imusolmukkeissa ei tapahtunut muutoksia. Kiertävien valkosolujen määrä laski hieman.

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