Effect of GnRH agonists on the thymus in female rats

Khalid M. Ataya\(^1\), Wael Sakr\(^2\), Charla M. Blacker\(^1\), Milton G. Mutchnick\(^3\) and Zuhair A. Latif\(^4\)

**Division of Reproductive Endocrinology and Infertility\(^1\), Department of Obstetrics and Gynecology, Departments of Pathology\(^2\) and Medicine\(^3\), Wayne State University, Detroit, MI, and Department of Clinical Immunology\(^1\), Abbott Laboratories, North Chicago, IL, USA

Abstract. The potential clinical applications of GnRH agonists are growing. We studied the effects of two GnRH agonists on the adult female rat thymus in 4 experiments. GnRH agonists administered sc and continuously significantly increased wet and dry thymic weights (absolute and relative). Thymic enlargement was related to the duration of treatment with GnRH agonists. The maximum increase in thymic weight occurred at approximately 18 days following initiation of treatment with GnRH agonists. Thymic enlargement does not appear to involve enhanced mitotic activity as measured by incorporation of tritiated thymidine into thymic tissue and thymic DNA. Histologic examination and computer-assisted morphometric analysis of thymuses indicated an increase in cortex to medulla ratio most pronounced at 10 and 18 days of GnRH agonist treatment. No consistent increases in splenic weight or bone marrow cell counts were observed. Thymosin alpha-1 but not thymosin beta-4 increased in GnRH agonist-treated rats. Thymic weight correlated negatively with ovarian and uterine weights, relative adrenal weight, serum estradiol, LH, and positively with thymosin alpha-1. Exogenous estrogen administration reversed GnRH agonist-induced thymic weight increase. Whether GnRH agonists have direct thymic effects remains to be determined.

The potential clinical applications of gonadotropin-releasing hormone and its agonists are growing. The agonists have been recommended for the treatment of prostatic carcinoma, precocious puberty, endometriosis, uterine fibroids, contraception, polycystic ovarian disease, and premenstrual syndrome (1). Moreover, there is a potential use of the agonists in the treatment of breast cancer and in the prevention of chemotherapy-induced gonadal failure (1,2).

Although GnRH agonists have been claimed to have no significant side effects, their actions on non-reproductive organ systems have not been fully evaluated. We have demonstrated that GnRH agonists increase body weight in female but not male rats (3). GnRH receptors have also been found in rat adrenals (4), but the physiological consequences are unknown. In our laboratory, Blacker et al. (5) demonstrated a significant increase in thymic weight following treatment of female rats with an agonist of GnRH. Moreover, GnRH agonists have been shown to increase thymic weight in young (6) and old male rats significantly more than orchietomy (p < 0.001) (7).

In this report, we studied the effect of GnRH agonists on adult female rat thymus, bone marrow and spleen. In addition, serum levels of thymosin alpha-1 (TA-1) and thymosin beta-4 (TB4), two hormone-like polypeptides found in thymus tissue, were measured.

**Materials and Methods**

**Animals**

Adult Sprague-Dawley female rats (mean body weight 243 ± 3.7 g, N = 13 for Experiment 1; 227 ± 2.9 g, N = 11 for Experiment 2; 304 ± 3.7 g, N = 38 for Experiment 3; 248 ±
2.5 g, N = 15 for Experiment 4 and 253 ± 39, N = 26 for Experiment 5) were fed rat chow and water ad libitum and housed 2/cage under controlled temperature (20–22°C) and light (14:10 LD). Vaginal smears were obtained daily and only rats demonstrating at least two consecutive normal estrous cycles were included. All female rats were sacrificed by decapitation in diestrus within one hour at the same time of the day in each experiment, usually between 09.00 and 10.00 h.

**Chemicals and administration methods**

[D-Ser (Bu')-6, Azgly-10]LHRH ethylamide (Zoladex, ICI 118630, GnRHz) pellets of lactide-glycolide copolymer degradable matrix were donated by Stuart Pharmaceuticals (Wilmington, Delaware), contained 1 mg of the agonists GnRHz to be released over 28 days and were implanted sc. When GnRHz pellets became unavailable to us [D-Leu-6, Des-Gly-10]GnR ethylamide (Leuprolide, Lupron, GnRH, obtained from Abbott Laboratories, North Chicago, IL) was dissolved in 0.9% NaCl and loaded into Alzet 2002 osmotic minipumps (Paolo Alto, CA) to release the agonist at a constant rate (5μg/day). The minipumps (life span: 2 weeks) were implanted under ether anesthesia on diestrus. Tritiated thymidine (³H-T) obtained from New England Nuclear (Boston, MA) (specific activity: 79.9 Ci/mmole) was diluted with an equal volume of 1.8% NaCl and loaded into Alzet osmotic minipumps model 2001 (life span: 1 week). Serum TA-1 and TB-4 were measured by enzyme-linked immunosorbert assays as previously described (8,9).

**Treatment regimens**

**Experiment 1.** Six rats were each implanted sc with 1 mg GnRHz pellet on diestrus under light ether anesthesia and the implantation was repeated every 23 days. Seven rats were used as controls. On day 50 of GnRHz treatment (≥ 2 days for controls) all rats were implanted sc with osmotic minipumps loaded with ³H-T and sacrificed 2 days later. At decapitation, trunk blood was collected and the following tissues and organs were isolated, cleaned, weighed, and combusted to determine ³H-T uptake (³HTU): thymus, ovary, uterus, duodenum, and blood. Bone marrow from the cavity of one femur was irrigated using Hanks’ balanced salt solution with 1% BSA and 0.15% EDTA. The marrow was then finely suspended, pushed through a 25-gauge needle and bone marrow nucleated cell counts were obtained using a ZM coulter counter with C-256 channelizer (Coulter Electronics, Hialeah, FL, USA). The bone marrow suspension was centrifuged; 0.4 ml of 38% concentrated HCl was added to the cell pellet. After dissolution 40 μl were used for combustion to evaluate bone marrow ³HTU. All tissues (approximately 40 mg) were processed through the biological material oxidizer (Hillsdale, NJ) as described earlier (10). The percent of ³HTU per mg of tissue was calculated as follows:

<table>
<thead>
<tr>
<th>Tissue cpm × recovery constant</th>
<th>Tissue sample weight</th>
<th>Total cpm released sc</th>
<th>Rat body weight</th>
</tr>
</thead>
</table>

where cpm stands for counts per minute for each tissue combusted and recovery constant is obatined by calibration of the oxidizer (usual value 1.0–1.3). Serum was separated and later assayed for TA-1 and TB-4.

**Experiment 2.** Six control and six GnRHz-implanted rats were either sham operated or implanted with GnRHz pellets sc as in Experiment 1 for 229 days. They were sacrificed 2 days after implanting the osmotic minipumps loaded with ³HT. Blood and tissues were handled as described for Experiment 1.

**Experiment 3.** Four groups of rats were implanted sc with GnRHz pellets. They were sacrificed 5 (7 rats), 10 (7 rats), 18 (7 rats), and 24 (6 rats) days after pellet implantation. Two control groups were sacrificed on days 5 (6 rats) and 24 (5 rats) after sham operation. Two days before sacrifice in diestrus, all rats were implanted with ³HT-loaded osmotic minipumps. Blood and other tissues were handled as described in Experiment 1. Since no significant differences in any of the parameters were obtained between the two control groups, they were combined for the purposes of statistical analysis.

**Experiment 4.** Eight rats each implanted with Alzet minipumps model 2002 loaded with GnRHa to release 5 μg per day sc. Seven control rats received sc minipumps loaded with 0.9% NaCl. On day 11, all GnRHa-treated rats were each implanted with 2001 Alzet minipumps loaded with ³TH, and sacrificed 2 days later in diestrus. Control rats were sacrificed in the diestrus closest to day 13 after implantation of GnRHa minipumps. Two days before sacrifice, all control rats received sc 2001 Alzet minipumps loaded with ³HT. A portion of the thymus was dried in an oven at 60°C and weighed serially until the weight decreased no further. DNA was extracted from portions of the thymus as described by Maniatis et al. (11). Incorporation of ³HT into thymic DNA was calculated, after counting radioactivity in a beta counter.

**Experiment 5.** Rats were subdivided into 4 groups: Groups 1 and 2 received GnRHa (5 μg/day by Alzet minipump model 2002) in diestrus. On day 6 of treatment, E2 silastic capsules, loaded as described by Legan et al. (12), were implanted sc in Group 1 while Group 2 were sham operated (empty capsules) on day 6; Groups 3 and 4 were implanted with Alzet minipumps (model 2002) loaded with 0.9% NaCl in diestrus. On day 6, Group 3 received E2 in silastic capsules, whereas Group 4 were implanted with empty capsules. All rats that were in persistent diestrus or estrus were sacrificed by decapitation on day 13 of minipump implantation. Serum, thymus, uterus and ovaries were handled as described earlier.
Histology and morphometric analysis.

Thymuses from Experiments 1 and 3 were fixed in formalin, processed, embedded in the flat position and sectioned (5 μm thickness). From each rat the section with the largest area, at approximately the middle of the block, was stained with hematoxylin and eosin. The magnified image of the thymus section was projected on a screen and the different thymic compartments (cortex, medulla and fibroadipose tissue) were delineated with different colors. A BioQuant image analysis system (Leitz, Switzerland) was then used to measure the areas. The cortical area was calculated by subtraction. For each rat thymus, the area occupied by each compartment was expressed as a percent of the total thymus section area. The means (± SEM) of the percentages for each compartment and the medullary to cortical area ratio were then calculated for each group and compared to control. The number of cells per unit cortical area was determined in some rats.

Statistics. Student's t-test, MANOVA, ANOVA, trend analysis, contrast analysis, Scheffe procedure and regression and correlation were used for statistical analysis as appropriate. A p < 0.05 was considered to indicate statistical significance.

Results

All rats treated with GnRH agonists entered persistent diestrus.

Experiments 1 and 2

The results of Experiments 1 and 2 are shown in Table 1. Significant increases in absolute and relative thymus weights and absolute spleen weight were observed in Experiment 1. A similar increase in absolute thymus weight was noted in Experiment 2, in which relative spleen weight was also increased.

Experiment 3

The changes in TA-1, TB-4, absolute and relative thymus weights, and spleen weight are shown in Fig. 1. There was a significant linear trend in TA-1, absolute and relative thymus weight (p < 0.001 for each). Trend analysis for TB-4 of thymus showed a quadratic trend (p < 0.001) and a linear trend (p < 0.005). No significant linear or quadratic trends in TB-4, TB-4, relative spleen weights were obtained. Thymic weight significantly and negatively correlated with parameters of ovarian function (ovarian ³HTU, r = −0.67, p < 0.01; ovarian weight, r = −0.53, p < 0.01; uterine weight, r = −0.74, p < 0.01; serum E₂, r = −0.41, p < 0.05; and LH, r = −0.53, p < 0.01). TA-1 also significantly and negatively correlated with parameters of ovarian function (ovarian ³HTU, r = −0.50, p < 0.01; ovarian weight, r = −0.43, p < 0.01; uterine weight, r = −0.65, p < 0.01; serum E₂, r = −0.32, p < 0.05 and LH, r = −0.50, p < 0.01.

Experiment 4

Table 2 shows that GnRHs significantly reduced

<table>
<thead>
<tr>
<th>Table 1. Effect of GnRHs on thymus weight, thymosins, ³HTU by the thymus, spleen weight, peripheral and bone marrow cell counts.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Number of rats</td>
</tr>
<tr>
<td>Thymus weight (mg)</td>
</tr>
<tr>
<td>Relative thymus weight (x10⁻³)</td>
</tr>
<tr>
<td>% ³HTU/mg thymus (x10⁻⁴)</td>
</tr>
<tr>
<td>TA-1 (ng/l)</td>
</tr>
<tr>
<td>TB4 (μg/l)</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
</tr>
<tr>
<td>Relative spleen weight (x10⁻³)</td>
</tr>
<tr>
<td>WBC count</td>
</tr>
<tr>
<td>Bone marrow cell count</td>
</tr>
<tr>
<td>Nucleated</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
</tr>
</tbody>
</table>

a: p < 0.05, b: p < 0.01, c: p < 0.001.
Effect of GnRHz (Zoladex) on the thymus and other parameters. The subtitle for each panel is at its top. In each panel, the number under each bar indicates the duration of exposure (days) to GnRHz. Distances between bars (on the x-axis) in each panel do not correspond with duration of treatment. a: p < 0.05; b: p < 0.01; c: p < 0.001.

Fig. 1.
**Table 2.**
Effect of GnRH agonist on thymus weights and tritiated thymidine incorporation in thymic DNA (13 days).

<table>
<thead>
<tr>
<th></th>
<th>GnRHa</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Wet thymus weight (mg)</td>
<td>456 ± 22</td>
<td>222 ± 21c</td>
</tr>
<tr>
<td>Relative thymus weight (×10⁻³)</td>
<td>1.63 ± 0.17</td>
<td>0.83 ± 0.08b</td>
</tr>
<tr>
<td>Dry thymus weight (mg)</td>
<td>98 ± 5</td>
<td>47 ± 5c</td>
</tr>
<tr>
<td>mg of DNA/thymus</td>
<td>5.4 ± 0.8</td>
<td>2.3 ± 0.5b</td>
</tr>
<tr>
<td>µg of DNA/mg thymus</td>
<td>12.1 ± 2.0</td>
<td>10.8 ± 2.4</td>
</tr>
<tr>
<td>cpm/mg DNA thymus</td>
<td>925 ± 188</td>
<td>3163 ± 931a</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>52 ± 3</td>
<td>22 ± 2c</td>
</tr>
</tbody>
</table>

*a*: p < 0.05,  
*b*: p < 0.050.01,  
*c*: p < 0.001.

[^3]HT incorporation into thymic DNA while significantly increasing absolute, and relative thymic weights. Dry thymic weight was also significantly increased in rats treated with GnRHa.

**Experiment 5**

The data are presented in Table 3. All rats that received E₂ were in persistent estrus; rats that received GnRHa alone were in persistent diestrus. MANOVA indicated that GnRHa increased body weight and thymus absolute and relative weights (p = 0.05–0.003, p < 0.001 and p < 0.001, respectively) and decreased absolute and relative uterine weights (p = 0.045 and p = 0.027, respectively). Exogenous E₂ reduced absolute and relative thymic (p < 0.001 and p < 0.001) and ovarian weights (p = 0.003, p = 0.004, respectively), and increased absolute and relative uterine weights (p < 0.001 and p < 0.001). Significant interaction was observed between GnRHs and E₂ on body weight gain (p = 0.001), absolute and relative thymus weights (p = 0.004 and 0.0042, respectively) and absolute and relative uterine weights (p = 0.003 and p = 0.002, respectively). One-way ANOVA and Scheffe procedure indicated that the effects of GnRHa on thymic and uterine weights can be completely reversed by E₂ (Table 3).

**Histology and morphometric analysis**

In Experiment 1 (52 days) there were no significant differences between control and GnRHt-treated rats in the percentages of the total thymic area oc-

**Table 3.**
Effect of GnRHa and E₂ on thymus, ovary and uterus weight and body weight gain.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GnRHa+E₂</th>
<th>GnRHa</th>
<th>E₂</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Number of rats</td>
<td>8</td>
<td>5</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>8.6 ± 3.2b¹</td>
<td>33.6 ± 7.7</td>
<td>11.0 ± 6.6b¹</td>
<td>0.4 ± 2.0b²</td>
</tr>
<tr>
<td>Thymus (mg)</td>
<td>8.6 ± 26b²</td>
<td>496 ± 31</td>
<td>168 ± 22b²</td>
<td>262 ± 18b²</td>
</tr>
<tr>
<td>Ovary (mg)</td>
<td>32 ± 5</td>
<td>61 ± 4a½</td>
<td>43 ± 5</td>
<td>56 ± 7</td>
</tr>
<tr>
<td>Uterus (mg)</td>
<td>626 ± 29b²</td>
<td>258 ± 42</td>
<td>575 ± 54b¹</td>
<td>484 ± 39b¹</td>
</tr>
<tr>
<td>Relative thymus weight (×10⁻³)</td>
<td>0.97 ± 0.10b²</td>
<td>1.76 ± 0.16</td>
<td>0.65 ± 0.08b²</td>
<td>1.00 ± 0.07b²</td>
</tr>
<tr>
<td>Relative ovary weight (×10⁻³)</td>
<td>0.12 ± 0.02</td>
<td>0.22 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.12 ± 0.02a½</td>
</tr>
<tr>
<td>Relative uterus weight (×10⁻³)</td>
<td>2.43 ± 0.11b²</td>
<td>0.90 ± 0.12</td>
<td>2.23 ± 0.22b²</td>
<td>1.88 ± 0.17b²</td>
</tr>
</tbody>
</table>

¹: Compared to Group 1, ₂: Compared to group 2, ³: Compared to Group 3. ¹: p < 0.05, using one-way ANOVA followed by Scheffe procedure. ²: p < 0.01, using one-way ANOVA followed by Scheffe procedure.
cuped by the cortex, medulla, fibroadipose connective tissue or medullary/cortical area ratio.

In Experiment 3, there was no significant difference in any of the thymic areas between the two control groups sacrificed on days 5 and 24 of treatment. These two control groups were thus combined. Only 3 of the thymuses in the group treated for 24 days with GnRHz were available for examination. All treatment groups were compared to control using contrast analysis within MANOVA. Overall, GnRHz treatment increased the proportion of thymic cortical area (p < 0.001) and decreased that of the medulla (p < 0.001). The mean (± SEM) percentages of thymic area occupied by the cortex for control rats and those treated for 5 (6 rats), 10 (6 rats), 18 (5 rats) and 24 days (3 rats) were 72 ± 1%, 77 ± 3% (p = 0.027), 81 ± 2% (p < 0.001), 82 ± 1% (p < 0.001) and 76 ± 3%, respectively. Significant linear (p = 0.028) and quadratic (p = 0.002) trends were observed. For the medulla, the percentages were 26 ± 1%, 22 ± 2% (p = 0.026), 18 ± 2% (p < 0.001), 17 ± 1% (p < 0.001) and 23 ± 3%, respectively. A quadratic trend (p = 0.001) was observed. Medulla to cortex ratios were 0.37 ± 0.2, 0.29 ± 0.04 (p = 0.051), 0.22 ± 0.03 (p < 0.001), 0.21 ± 0.02 (p < 0.001) and 0.30 ± 0.05, respectively. Linear (p = 0.046) and quadratic (p < 0.002) trends were observed. For fibroadipose tissue, the percentages for different groups were not different, all being around 1%. The number of cells per unit area in 10 randomly chosen cortical fields in each of 2 control rats, 2 GnRHz-treated rats (18 days) and 2 GnRHz-treated rats (24 days) was examined. No significant differences in cell density were obtained.

Discussion

GnRH agonists consistently increased thymus weight in all five experiments. The increase is not related to a higher water content, since dry weights were also increased with GnRH agonist therapy. On microscopic examination, the cortex area increased with a reduction in medulla to cortex ratio. Exogenous estradiol can prevent thymic enlargement.

Thymic enlargement may be due to enhanced cellular proliferation or to alterations in the process of entry, maturation and exit of thymic lymphocytes. There was an initial acceleration of 3HTU/mg of thymus on day 10, consistent with the results of Screpanti et al. (13) and Gulino et al. (14), followed by reduction on day 24. Thymus weight, however, continued to increase beyond day 10. This suggests that intrathymic cellular proliferation, as measured by 3THU/mg of thymus, does not appear to be the primary factor leading to thymic enlargement. This conclusion is further supported by the findings of Experiment 4, where thymic mitotic activity, as measured by 3HT incorporation into DNA, was reduced in the group with larger thymuses that received GnRH agonist for 13 days (Table 2). Similar reduction in 3HT incorporation into thymic DNA has also been observed in male rats in our laboratory (unpublished data).

The mechanisms of thymic enlargement following GnRH agonist treatment remain unclear. Direct effect at the thymic level have not been ruled out. The presence of GnRH receptors in thymic tissue has not been defined. Recent reports, however, indicate that GnRH agonists induce disturbances in peripheral microcirculation (15). This suggests that lymphocyte entry and exit from the thymus may be modified by GnRH agonists. Alternatively, in our rat model, it is possible that GnRHa-induced thymic enlargement may be mediated by hypoestrogenemia (16) induced by 'medical oophorectomy' effects of GnRH agonists. We have measured serum E2 in rats of Experiment 3 (10) and found it to be low. Serum E2 inversely correlated with absolute and relative thymus weight.

Other experiments in our laboratory (17) demonstrated that thymic enlargement induced by surgical ovariectomy (17.6 ± 1.2 mg of thymus per 100 g of body weight) was significantly less pronounced than that induced by GnRH agonist (22.0 ± 1.9, p < 0.05). Both were higher than control (14.2 ± 1.6). Thymic weight in ovariectomized rats treated with GnRH agonist was 20.8 ± 1.7. These data suggest that thymic enlargement following GnRH agonist treatment may not be solely due to 'medical oophorectomy' effects alone. On the other hand, exogenous E2 completely inhibited GnRHa-induced thymic enlargement (Table 3).

The effects of gonadectomy on the thymus have been studied rather extensively. In rodents, Castro (18) and Chiodi (19) observed that maximum difference in thymic weight occurred at 30—40 days after gonadectomy. The differences became gradually smaller thereafter. Similarly, in our experiments, thymic weight in GnRHz-treated rats more than doubled by day 18 of treatment, was less than 2-fold higher at 52 days and was approximately
50% higher than control at 229 days of treatment. Similar trend was observed in the cortex-medullary area ratio.

Our findings of increased rat TA-1 (Fig. 1) associated with hypoestrogenemia following GnRH agonist treatment are consistent with the findings of Hirokawa et al. (20) and Allen et al. (21). The physiologic relationship between TA-1 elevation and thymic enlargement remains unclear. Thymus weight, both absolute and relative, strongly correlated with TA-1, but not TB-4. Why TA-1 showed obvious significant increases on days 18 and 24 of treatment with GnRH (Fig. 1), but not on days 52 and 229 (Table 1) is not clearly understood. The discrepancy may be related to the duration of treatment with GnRH agonist. This would fit the same pattern of thymic weight changes we observed following GnRH agonist treatment and that observed by others following surgical gonadectomy (18,19). The pattern involves a significant initial increase that reaches a peak and gradually disappears later (18,19). On the other hand, the known age-related changes in TA-1 (22) may have played a role in the observed alterations in TA-1.

While the effects of ovarian factors on the thymus are well documented, thymic factors can also affect ovarian structure and function (23-28). Females with thymic aplasia also have dysgenetic ovaries (23). We have demonstrated the inhibition of loss of ovarian follicles in rats treated with GnRH in Experiments 1 and 2 (25). Whether this is mediated by thymic changes is not clear.

In conclusion, GnRH agonists increase thymic weight in female rats and this can be prevented by E2. The time-related changes in thymic weights, TA-1, TB-4 and 3HTU/mg of thymus have been defined. Further studies are needed to clarify the mechanism of these effects, and the interrelation between the thymus, ovary and hypothalamic-pituitary axis.

Acknowledgments

We wish to thank Debbie Paduchowski for her secretarial support, Drs Kamran Moghissi and Robert Sokol for their encouragement and support, and F. Weller for performing thymosin assays.

References


Received March 28th, 1989.
Accepted September 4th, 1989.

Dr Khalid M. Ataya,
Hutzel Hospital,
4707 St. Antoine,
Detroit, MI 48201,
USA.