

# The Effects of Gonadotropin-Releasing Hormone Immunization and Recombinant Follicle-Stimulating Hormone on the Leydig Cell and Macrophage Populations of the Adult Rat Testis

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**ABSTRACT:** The objective of this study was to investigate the role of the gonadotropins and, in particular follicle-stimulating hormone (FSH) in maintaining the Leydig cell and macrophage populations of the adult rat testis. Adult male Sprague-Dawley rats received a gonadotropin-releasing hormone (GnRH) immunogen for a period of 12 weeks in order to induce a selective deficiency in luteinizing hormone (LH) and FSH. Recombinant human FSH was then administered for 7, 14, and 21 days and macrophages and Leydig cells per testis quantified using the "optical disector" method. After GnRH immunization, Leydig cell and macrophage numbers were reduced by 18% and 68%, respectively, compared with normal controls, resulting in an increase in the ratio of Leydig cells to macrophages from 4:1 to 9:1. Leydig cells regressed morphologically following GnRH immunization, and macrophage mean nuclear diameter was

significantly reduced. Administration of FSH did not restore the numbers of either cell type; however, FSH did increase macrophage nuclear size. Eosinophils and mast cells were also found sparsely scattered throughout the interstitium after GnRH immunization and persisted in the FSH-treated animals. The results of this study indicate that in the adult rat: 1) both Leydig cell and macrophage numbers are reduced in the gonadotropin-deficient testis; 2) FSH has no effect on the number of either cell type in the absence of LH; and 3) testicular macrophage activity, as indicated by nuclear size, is stimulated by FSH, either directly or indirectly.

Key words: Stereology, morphometry, optical disector, leucocytes.

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The interstitium of the rat testis contains a relatively high density of resident macrophages compared with other connective tissues (Miller et al, 1983). Within the adult rat interstitium, macrophages and Leydig cells are consistently found in a ratio of approximately four Leydig cells to every macrophage (Hardy et al, 1989; Gaytan et al, 1994e). Extensive specialized cell junctions between the testicular macrophages and Leydig cells are frequently observed (Miller et al, 1983), and numerous studies have demonstrated functional interactions between these two cell types (see review by Hutson, 1994).

There have been many studies on the role of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the development of the interstitial tissue of the testis, although fewer such studies have examined the maintenance of this tissue in the adult. There is clear evidence that LH is involved in proliferation and maturation of

Leydig cells during puberty and after treatment with the Leydig cell cytotoxin, ethylene dimethane sulfonate (EDS) (Molenaar et al, 1986; Teerds et al, 1989a,b,c). In the immature hypophysectomized rat, FSH stimulates Leydig cell numbers by increasing proliferation and differentiation of their progenitor cells, presumably mediated via the Sertoli cells (Kerr and Sharpe, 1985; Teerds et al, 1989a). In contrast, FSH is not required for recovery of Leydig cells in mature rats after EDS treatment (Molenaar et al, 1986). In adult rats depleted of gonadotropins by hypophysectomy or subcutaneous steroid implants, Leydig cell number has been reported as being not significantly different from normal (Keeney and Ewing, 1990; Russell et al, 1992); however, Gaytan et al (1994d) detected a small decrease in Leydig cell number after hypophysectomy.

Macrophage numbers increase in parallel with Leydig cell numbers during testicular development (Hardy et al, 1989; Hutson, 1990; Raburn et al, 1993). Human chorionic gonadotropin (hCG) increases the mitotic index of macrophages in the neonatal testis, suggesting a role for gonadotropic hormones in the establishment of the testicular macrophage population (Raburn et al, 1991, 1993). In long term hypophysectomized adult rats, substantial

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losses of testicular macrophages have been reported (Dombrowicz et al, 1992; Gaytan et al, 1994e). Prolactin (Prl) and growth hormone (GH) can partially restore macrophages posthypophysectomy (Dombrowicz et al, 1992; Gaytan et al, 1994d,e); however, combined treatment with human LH and FSH completely restores macrophage numbers (Gaytan et al, 1994d). The respective roles of LH and FSH in this restoration are unknown.

Within the testicular interstitial tissue, macrophages undergo a process of morphological and functional maturation, which is accompanied by an increase in nuclear size (Wang et al, 1994). Yee and Hutson (1983) demonstrated that testicular macrophages, but not peritoneal macrophages, express high affinity FSH receptors, consistent with the development of testis-specific macrophage functions (Hutson, 1994). *In vitro*, FSH stimulates the cellular metabolism of testicular macrophages by increasing the production of cyclic adenosine monophosphate (cAMP), lactate, and RNA and polypeptide synthesis (Yee and Hutson, 1983, 1985). Moreover, evidence of an essential role for the testicular macrophages in Leydig cell development has been provided by Gaytan et al (1994a,b,c, 1995), who demonstrated that establishment of the Leydig cell population of the pubertal rat and, in the EDS-treated adult rat, is prevented when testicular macrophage numbers are depleted.

In a previous study, adult rats were actively immunized against gonadotropin-releasing hormone (GnRH), and FSH was then replaced by treatment with human recombinant FSH (rhFSH) (McLachlan et al, 1995). This experimental model has the advantage over the hypophysectomized model of selectively eliminating the gonadotropins, while the use of a recombinant source of FSH eliminates the potential problem of LH contamination associated with purified gonadotropins. Changes in spermatogenesis and testosterone secretion have already been reported (McLachlan et al, 1995). In the present study, these tissues have been re-examined in order to assess the effects of gonadotropin depletion and specific FSH replacement on the regulation of Leydig cell and macrophage number and function in the adult rat.

## Materials and Methods

### Animals

The testicular tissue utilized was collected during a previous study by McLachlan et al (1995). Adult (90–110 days old, 350–450 g) Sprague-Dawley rats from the Monash Central Animal House (Clayton, Melbourne, Australia) were maintained at 20°C in a fixed 12 hour light:12 hour dark light cycle with free access to food and water.

### GnRH Immunization

Experimental animals received 100 µg of GnRH immunogen (BA-17, Biotech Australia P/L, Sydney, Australia), incorporating an adjuvant free of mycobacterial components every 4 weeks (McLachlan et al, 1994). Control animals received adjuvant only. Rats were assessed at week 12 by manual palpation of the testes under anesthesia. Animals exhibiting a testicular volume <0.55 ml were selected for the study and given a final GnRH immunization at week 12.

### FSH Administration

Lyophilized rhFSH (Gonal-F, Serono Australia, Sydney, Australia) was reconstituted in sterile water to either 50 IU/ml or 250 IU/ml, kept at 4°C, and used within 1 day. Gonadotropin-releasing hormone-immunized animals received either 10 or 50 IU rhFSH/kg daily sc for 7 ( $n = 6$  animals), 14 ( $n = 6$ ), 21 ( $n = 6$ ), or 28 ( $n = 6$ ) days, while untreated control ( $n = 10$ ) and immunized control ( $n = 12$ ) animals received an equivalent volume of normal saline. Animals were killed 6 hour after the final rhFSH injection. Immunized control rats were separated into two equal groups and killed at the beginning of rhFSH administration (day 0,  $n = 6$ ) and at the time of the final rhFSH injection (day 28,  $n = 6$ ). Antibodies to rhFSH were first detected after the 2 week, and animals displaying antibodies were eliminated from the original study (McLachlan et al, 1995). As a result, all animals at the 28-day time point were eliminated. There was no apparent difference in testicular weight, germ cell numbers, stereological parameters, or serum inhibin levels between the 10- and 50-IU-dose groups at the 1 week time point (McLachlan et al, 1995). Accordingly, the data for both doses were pooled to give a single rhFSH-treatment group for each of the remaining time periods, that is 7 ( $n = 12$  animals), 14 ( $n = 7$ ), and 21 days ( $n = 4$ ). Moreover, there was no difference in any Leydig cell or macrophage parameter measured in the two groups of immunized control rats in the present study; hence, these groups were recombined for analysis.

### Tissue Preparation

Animals received heparin (porcine mucous, 1,000 IU, sc) 0.5–2 hours prior to perfusion. Animals were anesthetized, the thoracic aorta was cannulated, and the vascular system was flushed with normal saline followed by Bouin's fluid. The left testis was removed, weighed, and sliced into a series of 2-mm-thick slabs orthogonal to the long axis of the testis. Three slabs per animal were selected by systematic uniform random sampling (Wreford, 1995), and one-half of each slab was selected for processing into hydroxyethyl methacrylate (Technovit 7150, Kulzer and Co., GmbH, Friedrichsdorf, Germany) according to the manufacturer's instructions. Blocks were oriented to give transverse sections of tubules, cut at 2 and 25 µm, stained with periodic acid Schiff's (PAS), and counterstained with hematoxylin.

### Estimation of Cell Number

The number of Leydig cells and macrophages was estimated using the optical disector method (Gundersen et al, 1988a; Wreford, 1995). Cells were counted in 25 µm sections by using a 100× oil immersion objective on an Olympus (Tokyo, Japan)

BH-2 microscope. The microscope image was captured using an F15 Panasonic (Osaka, Japan) videocamera and input to an Amiga 2000 computer (Commodore Business Machines P/L, Sydney, Australia) equipped with an Impact Vision-24 professional videoadapter (Great Valley Products Inc., King of Prussia, Pennsylvania). The Medicosoft GRID (v.1.2) software package (Graf-Itidata, Silkeborg, Denmark) was used to generate a set of two (area = 980  $\mu\text{m}^2$ /frame) or 4 (area = 650  $\mu\text{m}^2$ /frame) unbiased counting frames (Gundersen, 1977; Gundersen et al, 1988b) that were superimposed on the image from the microscope. The number and area of frames were determined to provide the most efficient counting regime. Fields were sampled using a systematic uniform random scheme generated using a motorized stage (Lang GMBH, Huttenburg, Germany) to eliminate any bias in their selection (Gundersen and Jensen, 1987). The step length in the x and y direction of the stage was selected to allow 100–200 macrophages to be counted per testis and ranged from 210  $\mu\text{m}$  to 550  $\mu\text{m}$ . A microcator, fitted to the microscope stage, was used to measure the movement of the stage in the z axis.

For each section, the upper surface was focused, and a guard area of the upper 5  $\mu\text{m}$  of the section was traversed to avoid any artefacts associated with the cut face. As the microscope was focused through the next 10  $\mu\text{m}$  of the section, cells were counted on the basis of their nuclei coming into sharp focus if they were within, or partially within, the unbiased counting frame, so long as no part of them touched or crossed the exclusion lines of the frame (Wreford, 1995). This same procedure was used to estimate mast cells and eosinophils, but these cells were too sparse to allow accurate quantitation with this sampling regime.

#### *Estimation of Macrophage Mean Nuclear Diameter*

The mean nuclear diameter of the macrophages in the number distribution (Wreford, 1995) was also estimated in 25  $\mu\text{m}$  sections using the optical disector to sample cells for measurement. Macrophage nuclei were brought into sharp focus on the computer screen, and the mean nuclear diameter for each macrophage was determined directly using the distance measurement facility of the Medicosoft Grid software. This was achieved by taking the arithmetic mean of the long and the short axes. The mean diameter of 30–50 systematically sampled nuclei was measured per animal.

#### *Photography*

Photographs of testicular tissue were obtained from 2  $\mu\text{m}$  sections on Fujichrome Velvia film (Fuji Photo Film, Tokyo, Japan) at ASA 32 using a Zeiss photomicroscope (Carl Zeiss, New York, New York) with a 40 $\times$  objective.

#### *Statistical Analysis*

Statistical comparisons were analyzed by analysis of variance (ANOVA) in conjunction with a Student-Newman-Keuls test using Sigmastat v.1.0 (Jandel Scientific, San Rafael, California). All data in both text and figures are reported as mean  $\pm$  SEM.

## **Results**

### *Cell Identification*

Leydig cells were identified by their flattened ovoid nuclei and characteristic punctate chromatin (Fig. 1a). A furrow running the length of one side of the nucleus was frequently observed in thick sections. Macrophages were identified by their indented or kidney-shaped nucleus with darkly stained peripheral chromatin (Fig. 1a). In control animals, the macrophage cytoplasm was PAS positive (Fig. 1a).

### *Qualitative Observations*

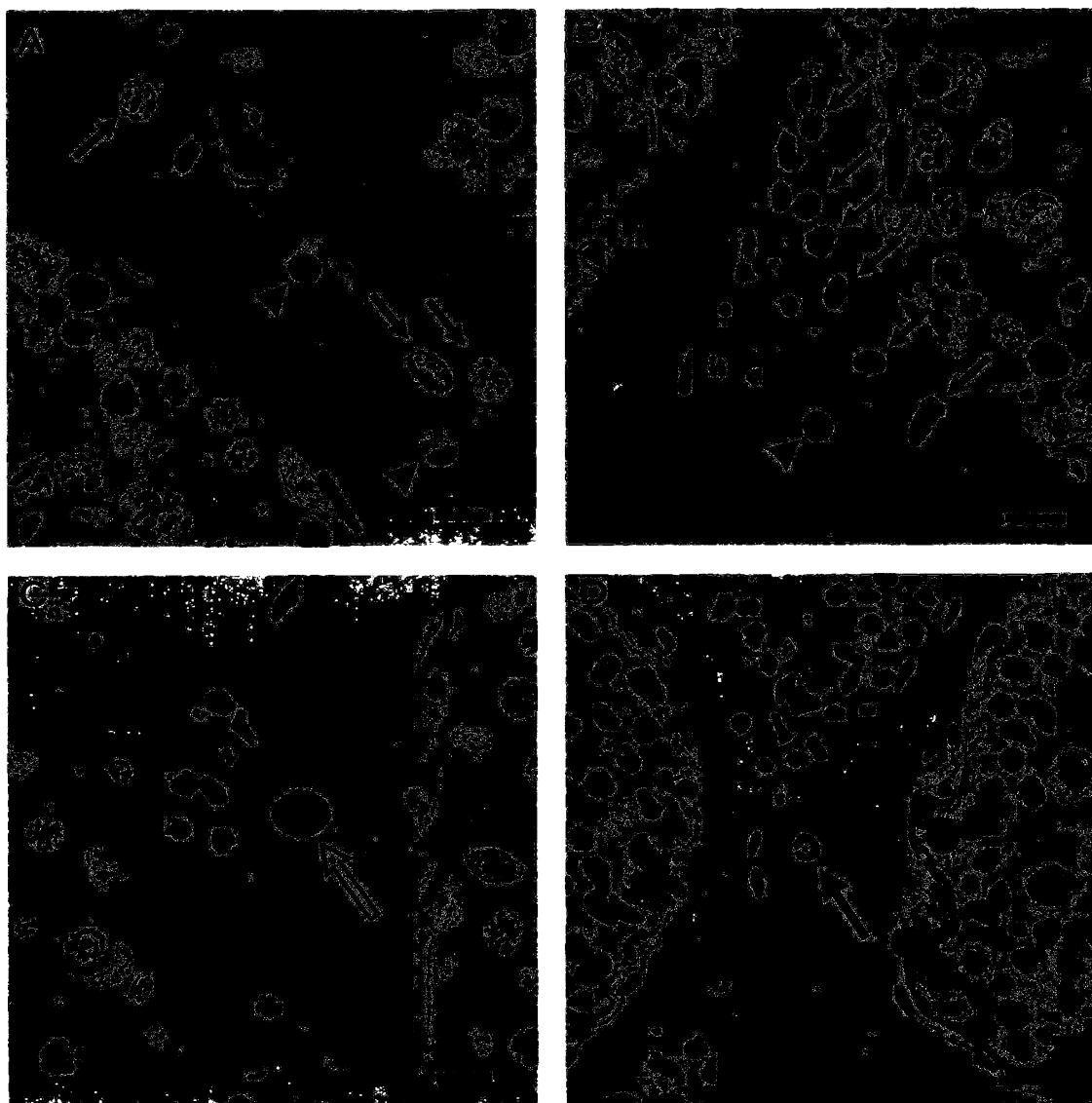
After GnRH immunization, the seminiferous tubules showed a marked reduction in diameter with germ cells restricted to the earliest stages (Fig. 1), as reported earlier (McLachlan et al, 1995). These changes were partially reversed on administration of rhFSH. Both the nuclei and cytoplasm of the Leydig cells were reduced in volume following GnRH immunization, but these cells could still be clearly identified by their characteristic nuclear morphology (Fig. 1b). Macrophage nuclear morphology did not change following GnRH immunization, but the cytoplasm no longer stained with PAS. No changes in Leydig cell and macrophage morphology or staining characteristics were seen in response to treatment with rhFSH.

Following GnRH immunization, mast cells containing intensely basophilic cytoplasmic granules (Fig. 1c) and occasional eosinophils, characterized by their distinct annular-shaped nuclei (Fig. 1d), were observed within the intertubular tissue. Treatment with rhFSH caused no observable change in these cells. Neither mast cells nor eosinophils were seen in the intertubular tissue of control testes, although numerous subcapsular mast cells were present in all testes.

### *Quantitative Studies*

Leydig cell numbers per testis tended to be reduced in GnRH-immunized groups (Fig. 2A), although this reduction reached statistical significance only in the GnRH animals treated with rhFSH for 21 days. Treatment with rhFSH did not restore Leydig cell number. When Leydig cell number in the control ( $n = 10$ ) versus the combined GnRH-immunized  $\pm$  FSH groups ( $n = 35$ ) were compared, a significant reduction of 18% ( $P < 0.001$ ) was apparent. Macrophage numbers per testis were reduced by approximately 68% in all GnRH-immunized groups (Fig. 2B), and rhFSH had no effect in restoring macrophage numbers. The ratio of Leydig cell to macrophage number was  $3.5 \pm 0.2$  in control animals. Following GnRH immunization, this ratio increased to  $9.3 \pm 0.6$ , and there was no significant change when rhFSH was given between 7 and 21 days (range 8.2–9.9).

After GnRH immunization, there was a significant re-



**FIG. 1.** Light micrograph of the interstitium of the adult rat testis. (a), Leydig cells (arrows) and macrophages (arrowhead) in untreated animals, (b), regressed Leydig cells (arrows) and macrophages (arrowhead) in animals treated with gonadotropin-releasing hormone (GnRH) immunogen for a period of 12 weeks, (c), interstitial mast cell (arrow) in animals treated with GnRH immunogen for a period of 12 weeks and recombinant human follicle-stimulating hormone (rhFSH) for 7 days, and (d), interstitial eosinophil (arrow) in animals treated with GnRH immunogen for a period of 12 weeks. Scale bar represents 10  $\mu\text{m}$ .

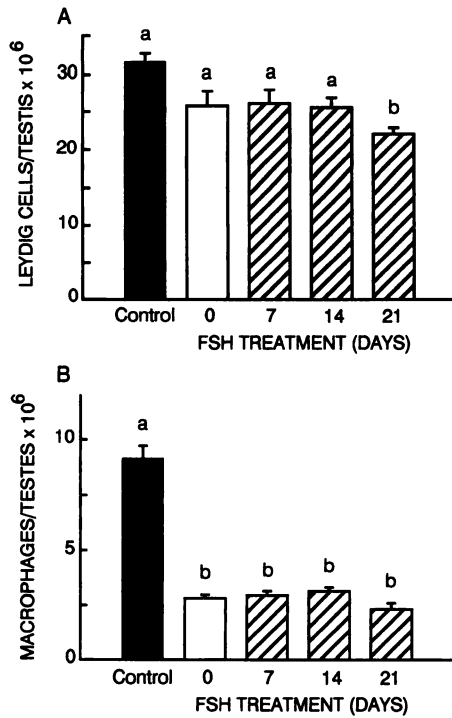
duction in mean nuclear diameter of macrophages from 5.7  $\mu\text{m}$  to 5.3  $\mu\text{m}$ , which was restored with administration of rhFSH at 7 and 14 days and significantly increased after 21 days of treatment (Fig. 3).

## Discussion

The data in the present study confirm a previous report in hypophysectomized rats that maintenance of both Leydig cell and macrophage numbers in the adult rat testis is dependent upon pituitary gonadotropins (Gaytan et al, 1994d). Macrophages are much more sensitive to gonad-

otropin withdrawal compared to Leydig cells. Since restoration of FSH had no effect on the number of either cell type in the absence of LH, this implies that the numbers of both cell types are regulated by LH in the adult.

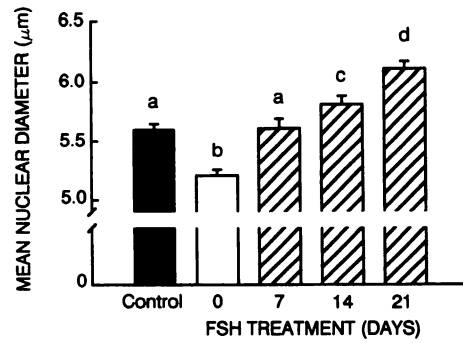
Leydig cells and macrophages were readily distinguished in both control and GnRH-immunized testes in 25- $\mu\text{m}$ -thick methacrylate sections when stained with PAS and hematoxylin. In thick sections, it was possible to visualize the entire nucleus, greatly facilitating the recognition of cells by their distinct nuclear morphologies. In addition to the striking regression of the seminiferous tubule following GnRH immunization (McLachlan et al, 1995), Leydig cell regression similar to that observed af-



**FIG. 2.** Effect of recombinant human follicle-stimulating hormone (rhFSH) treatment (10 IU or 50 IU/kg body weight sc daily) over time (days) of gonadotropin-releasing hormone (GnRH)-immunized adult rats on: (A), Leydig cell number per testis and (B), macrophage number per testis. Results for (A) and (B) are mean  $\pm$  SEM. b, significant ( $P < 0.001$ ) with respect to untreated animals.

ter hypophysectomy was also apparent (Keeney and Ewing, 1990; Russell et al, 1992; Gaytan et al, 1994d). Leydig cell cytoplasm and nuclear size were reduced; however, the characteristic nuclear shape and chromatin distribution were maintained. On the other hand, macrophage appearance changed little after GnRH immunization, except that PAS reaction was no longer observed in the cytoplasm, possibly reflecting a loss of the ability of testicular macrophages to accumulate glycoconjugates in the gonadotropin-deficient rat testis, as previously observed in the ageing mouse testis (Tanemura et al, 1993).

Estimates of Leydig cell and/or testicular macrophage number in normal rats have been carried out using a variety of techniques (Mori and Christensen, 1980; Kerr and Sharpe, 1985; Kerr et al, 1987; Mendis-Handagama et al, 1987; Hardy et al, 1989; Keeney et al, 1990; Raburn et al, 1991; Russell et al, 1992; Gaytan et al, 1994b,d; Wang et al, 1994). These earlier studies have reported estimates from 22 to 34 million Leydig cells/testis and from 7.5 to 9.0 million macrophages/testis, indicating a ratio of four Leydig cells to each macrophage. The optical disector used in the present study is an efficient stereological method for the estimation of cell number that is independent of assumptions with regard to particle size or shape



**FIG. 3.** Effect of recombinant human follicle-stimulating hormone (rhFSH) treatment (10 IU or 50 IU/kg body weight sc daily) over time (days) of gonadotropin-releasing hormone (GnRH)-immunized adult rats on the mean nuclear diameter of testicular macrophage ( $\mu\text{m}$ ). Results are mean  $\pm$  SEM. b and c, significant ( $P < 0.05$ ) with respect to untreated animals.

(Gundersen, 1986; Wreford, 1995). Using this method, cell number estimates were in good agreement with our own previous estimates using frozen sections (Wang et al, 1994) and earlier estimates (Mori and Christensen, 1980; Kerr and Sharpe, 1985; Kerr et al, 1987; Hardy et al, 1989; Keeney et al, 1990; Russell et al, 1992). While the data in the present study are consistent with previous studies (Hardy et al, 1989; Keeney and Ewing, 1990) using the physical disector (Sterio, 1984), the advantage of the optical disector is that the problem of obtaining perfect register of consecutive physical sections is eliminated.

Testicular macrophage and Leydig cell numbers have not been determined previously in the GnRH-immunized model. However, the 18% reduction in Leydig cell number in the present study is consistent with the observations of Keeney and Ewing (1990) and Gaytan et al (1994d) after hypophysectomy. These data suggest that maintenance of Leydig cell number in the adult rat is partially dependent upon gonadotropin support, and as rhFSH treatment for up to 21 days did not restore Leydig cell numbers, this suggests that LH is primarily responsible. In studies on Leydig cell development in the immature rat and in the EDS-treated rat, LH appears to have only a marginal effect on Leydig cell proliferation, although it is essential in Leydig cell differentiation and maintenance of the differentiated state (Benton et al, 1995).

Testicular macrophages were severely depleted after GnRH immunization, and the ratio of Leydig cells to macrophages increased from 4:1 to 9:1, a figure consistent with that found in the GH- and Prl-treated long term hypophysectomized rat (Dombrowicz et al, 1992; Gaytan et al, 1994e). The failure of rhFSH to restore testicular macrophage numbers in the GnRH-immunized rat suggests LH, and hence the Leydig cell, is responsible for macrophage maintenance in the adult, as appears to be the case during development (Hutson, 1994). Earlier evidence

for a linkage between Leydig cell function and macrophage number in the adult comes from data derived from Leydig cell depletion studies employing EDS (Wang et al, 1994). After an initial increase in macrophage numbers in response to the wave of Leydig cell death, macrophage numbers decline below normal levels in spite of the presence of elevated gonadotropin levels. Macrophage numbers eventually return following differentiation of a new population of Leydig cells. If gonadotropin levels are suppressed with testosterone implants after EDS treatment, however, the Leydig cells do not recover and macrophage numbers remain low (approximately 50% of normal). Hence, there appears to be no correlation between macrophage number and gonadotropin levels themselves, indicating that it is the presence of functional Leydig cells that is responsible for maintaining macrophage numbers.

In addition to its effects on cell number, GnRH immunization significantly reduced the mean nuclear diameter of testicular macrophages to 5.3  $\mu\text{m}$ , which was then restored by rhFSH treatment and actually increased above control levels to 6.2  $\mu\text{m}$  at 21 days. This data can be compared to the difference between the nuclear diameter of monocytes within the interstitium (5.2  $\mu\text{m}$ ) and that of more mature resident testicular macrophages (6.0  $\mu\text{m}$ ) identified immunohistochemically in frozen testicular sections (Wang et al, 1994). Together, these data suggest that the activity, and possibly the maturation, of the testicular macrophages is stimulated by FSH, either directly via FSH receptors (Yee and Hutson, 1983) or indirectly by local factors from the seminiferous tubule.

Immunization against GnRH increased the number of cells normally associated with inflammation in the interstitium. Mast cells, normally located just deep to the tunica albuginea in the rat, were found scattered throughout the intertubular tissue. Eosinophils, rarely if ever seen in the testis in the absence of infection or autoimmune orchitis (Kohno et al, 1983; Nistal et al, 1986), also appeared, although their number (approximately 20,000/testis) was insufficient to enable a reliable estimate. These increases were not affected by rhFSH administration. This invasion by inflammatory cells is consistent with data from other studies where the pituitary-testis axis has been disrupted, such as by EDS treatment (Jackson et al, 1986; Wang et al, 1994), hypophysectomy (Gaytan et al, 1990, 1992), macrophage depletion by administration of dichloromethylene diphosphonate-containing liposomes (Gaytan et al, 1990, 1992), and hCG-induced hyperstimulation (Bergh et al, 1993). Since the mechanisms involved are unknown, further investigation into the local control of inflammatory responses in the testis is needed.

In summary, these data, along with the results of previous studies, imply that LH is the main regulator of macrophage numbers in the adult testis. This regulation most likely occurs via the action of LH upon Leydig cell func-

tion. In the absence of LH, FSH has no effect on macrophage numbers. However, the maturation or functional activity of macrophages in the adult testis appears to be stimulated either directly or indirectly by FSH. Finally, withdrawal of gonadotropins leads to an increase in inflammatory cells in the testis that may be related to the decrease in macrophages or Leydig cell activity.

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