

Effect of Chorionic Gonadotropin and Flutamide on Leydig Cell and Macrophage Populations in the Testosterone-Estradiol-Implanted Adult Rat

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ABSTRACT: The objective of this study was to investigate the role of androgens and nonandrogenic Leydig cell products in maintaining Leydig cell and macrophage numbers in the testis of the adult rat. Adult male Sprague-Dawley rats received Silastic implants containing testosterone and estradiol (T-E) in order to suppress endogenous luteinizing hormone (LH) for 9 weeks. After T-E treatment, Leydig cell and macrophage numbers, quantified using the optical disector approach, were reduced by 40 and 60%, respectively, compared with controls. Administration of human chorionic gonadotropin (hCG) for a period of 10 days restored Leydig cell numbers to control levels, and macrophage numbers were partially restored. Administration of the antiandrogen, flutamide, in combination with hCG treatment in T-E implanted animals prevented the restoration of Leydig cell numbers but did not prevent the recovery of macrophage num-

bers. In the T-E-implanted animals, there was a decrease in testicular macrophage nuclear size, which was not restored by either hCG or hCG plus flutamide treatment. The results of this study support the hypothesis that LH is the main pituitary regulator of both Leydig cell and macrophage number in the adult rat testis and further indicate that androgens are responsible for maintaining Leydig cell numbers and/or differentiation, but nonandrogenic Leydig cell factors are primarily responsible for controlling macrophage numbers. Testicular macrophage function, as indicated by nuclear size, does not appear to be influenced by LH or testosterone in the adult rat.

Key words: Testosterone-estradiol implants, stereology, optical disector, morphometry, macrophage nuclear diameter, testicular interstitium.

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Leydig cells and macrophages are morphologically and functionally associated within the testis (see review by Hutson, 1994). The adult population of both cell types is established at the time of puberty (Hardy et al, 1989; Benton et al, 1995), and a ratio of approximately four Leydig cells to every macrophage is consistently found in the normal adult rat testicular interstitium (Niemi et al, 1986; Mendis-Handagama et al, 1988; Hardy et al, 1989; Gaytan et al, 1994). There have been many studies on the development of the interstitial tissue of the testis, but few such studies have examined the hormonal maintenance of this tissue in the adult. We previously reported a significant reduction in both Leydig cell and macrophage numbers in the adult rat testis after withdrawal of the gonadotropins by gonadotropin-releasing hormone (GnRH) immunization (Duckett et al, 1997), which was consistent with the previously observed effects of hypophysectomy

(Gaytan et al, 1994). Treatment of GnRH-immunized rats with human recombinant follicle-stimulating hormone (FSH) in the absence of luteinizing hormone (LH) had no effect on the number of either cell type (Duckett et al, 1997), indicating that LH is the gonadotropin primarily responsible for maintaining these cells in the adult testis.

Both FSH and LH are required during development for the pubertal increase in Leydig cell numbers (Kerr and Sharpe, 1985a; Teerds et al, 1989a), although only LH is required for proliferation and maturation of Leydig cells in the adult after their destruction by ethane dimethane sulfonate (EDS) treatment (Molenaar et al, 1986; Teerds et al, 1989b,c). In addition, testosterone appears to be necessary to stimulate the differentiation of the mature Leydig cell from the immature Leydig cell precursors (Hardy et al, 1990; Misro et al, 1992, 1993; Benton et al, 1995).

Macrophage numbers increase in parallel with Leydig cell numbers during pubertal development (Hardy et al, 1989). Human chorionic gonadotropin stimulates the proliferation of macrophages in the neonatal testis, suggesting a role for LH in the establishment of the testicular macrophage population (Raburn et al, 1991, 1993). This proliferation of macrophages in the immature rat is not affected by the administration of the antiandrogen, Ca-

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sodex, indicating that androgens are not involved (Raburn et al, 1993). In long-term hypophysectomized adult rats, substantial losses in testicular macrophages occur (Gaytan et al, 1994). Combined treatment with human LH and FSH completely restores macrophage numbers in hypophysectomized adult rats (Gaytan et al, 1994), and we have established that FSH alone cannot restore macrophage numbers after gonadotropin withdrawal (Duckett et al, 1997). This decline appears to be linked to the loss of Leydig cell function, since macrophage numbers also fall following ablation of Leydig cells by EDS treatment (Wang et al, 1994). The mechanism and putative Leydig cell-derived mediator of LH action on the macrophages remains to be identified.

In a previous study (Meachem et al, 1997), adult rats received testosterone-estradiol (T-E) implants to induce a state of LH and testosterone deprivation (Ewing et al, 1977) within the testis. Leydig cells were subsequently stimulated with hCG treatment alone or in combination with the antiandrogen, flutamide, to determine the effects of androgens and nonandrogenic products of Leydig cells on spermatogenesis. In the present study, the tissues have been re-examined in order to assess the effects of LH withdrawal, hCG replacement, and androgen blockade on the regulation of Leydig cell and macrophage number and function in the adult rat interstitial tissue.

Materials and Methods

Animals

The testicular tissue utilized was collected during a previous study by Meachem et al (1997). Adult (75–90 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.

Experimental Procedure

Implants were prepared by packing polydimethylsiloxane tubing (o.d., 3.18 mm; i.d., 1.98 mm; Dow Corning, Midland, Michigan) with testosterone or estradiol-17 β (Sigma, St Louis, Missouri) powder and sealing the ends with medical adhesive silicone type A (Dow Corning) as described in Meachem et al (1996). Human chorionic gonadotropin (Pregnyl, Organon, Cambridge, UK) was prepared in normal saline. The androgen receptor antagonist, flutamide (Sigma), was prepared in a vehicle consisting of equal volumes of absolute ethanol and sesame oil (Imperato-McGinley et al, 1992).

Treated animals received subcutaneous (s.c.) implants of testosterone (3 cm long) and estradiol (0.4 cm long) for a period of 9 weeks (T-E treatment). Animals were assigned to one of four experimental groups: 1) vehicle-treated controls ($n = 5$) receiving ethanol:sesame oil vehicle injection only (1.0 ml/kg, daily, s.c.) for 10 days, 2) T-E-implanted vehicle control rats ($n = 5$) receiving vehicle injection only for 10 days, 3) T-E-implanted rats ($n = 6$)

receiving hCG (1.25 IU/kg, daily, s.c.) and vehicle for 10 days, and 4) T-E-implanted rats ($n = 6$) receiving hCG and flutamide (100 mg/kg, daily, s.c.) dissolved in vehicle for 10 days.

Hormone measurements have been reported in the previous study of Meachem et al (1997). Total testicular testosterone levels were suppressed by T-E treatment to below the level necessary to maintain spermatogenesis. Treatment with hCG restored testosterone to approximately 12% of vehicle-treated control levels, adequate to maintain normal spermatogenesis, but this restoration was prevented by flutamide. Serum FSH was suppressed to 51% of vehicle-treated controls by T-E treatment and returned to normal levels following hCG treatment for 10 days but not in animals receiving flutamide over the same time frame.

Tissue Preparation

Animals received an injection of heparin (porcine mucous, 1,000 IU, s.c.) 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 (Gundersen and Jensen, 1987; 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 μ m and were stained with periodic acid-Schiff's (PAS) and counterstained with hematoxylin.

Estimation of Cell Number and Macrophage Mean Nuclear Diameter

The number of Leydig cells and macrophages were estimated in 25 μ m sections using the optical disector method (Gundersen et al, 1988; Wreford, 1995) as previously described (Duckett et al, 1997). Macrophage mean nuclear diameter was estimated as previously described (Duckett et al, 1997). The criteria for Leydig cell and macrophage identification were strictly defined using their characteristic nuclear morphologies as previously reported (Duckett et al, 1997). Mature Leydig cells were identified by their flattened ovoid nuclei, characteristic punctate chromatin, and frequently observed nuclear furrow. In hCG-treated groups, a subset of Leydig cells were observed, which were characterized by a rounded nucleus with a more prominent single nucleolus and less punctate peripheral heterochromatin. These cells displayed the characteristic nuclear morphology of immature or fetal-type Leydig cells and newly formed Leydig cells observed in testes recovering after EDS treatment (Kerr and Sharpe, 1985a,b; Kerr et al, 1987). Macrophages were identified by their indented or kidney-shaped nucleus with darkly stained peripheral chromatin. In control animals, the macrophage cytoplasm was PAS-positive (Duckett et al, 1997).

Photography

Photographs of testicular tissue were obtained from 2 μ m sections on Fujichrome Velvia film (Fuji Photo Film, Tokyo, Japan) at ASA 32 using a Leitz Diaplan photomicroscope fitted with an orthomat camera (Leitz, Germany).

Statistical Analysis

Data was analyzed by analysis of variance in conjunction with a Student-Newman-Keuls *post hoc* test using Sigmatat v1.0 (Jandel Scientific, San Rafael, California). Prior to analysis, data were log-transformed in order to equalize the variance between experimental groups. All data are reported as mean \pm SEM.

Results

Qualitative Observations

Mature Leydig cells and macrophages were frequently observed in vehicle-treated control animals (Fig. 1A). After T-E implantation, the seminiferous tubules were reduced in diameter with no elongated spermatids. These tubule changes were partially reversed after administration of hCG, and elongated spermatids were frequently observed. When hCG was given in combination with flutamide, the conversion of germ cells to progressively later stages was again lost. Both the nuclei and cytoplasm of the Leydig cells were reduced in volume following T-E implantation, but these cells could still be clearly identified by their characteristic nuclear morphology (Fig. 1B). After 10 days of hCG administration, the characteristic appearance of the Leydig cell was restored (Fig. 1C). In addition, immature Leydig cells, as well as cells with a transitional nuclear morphology, were observed in the interstitium (Fig. 1C).

After treatment with hCG in combination with flutamide, most Leydig cells remained regressed; however, these cells exhibited nuclear morphologies characteristic of both adult and immature Leydig cells (Fig. 1D). Neither macrophage nuclear nor cytoplasmic morphology appeared to change after T-E treatment nor was there any apparent change in their morphology after treatment with hCG alone or in combination with flutamide.

Following T-E treatment, mast cells and occasional eosinophils were observed within the intertubular tissue (data not shown). Treatment with hCG alone or in combination with flutamide caused no observable changes in these cells. Neither mast cells nor eosinophils were seen in the intertubular tissue of vehicle-treated control testis.

Quantitative Studies

Adult and immature nuclear morphologies were grouped for counting of total Leydig cell numbers. The number of Leydig cells per testis was reduced by 40% in T-E-implanted animals compared with vehicle-treated control animals (Fig. 2A). Treatment with hCG for 10 days restored Leydig cell numbers to vehicle-treated control levels; however, this recovery was prevented by coadministration of the androgen receptor antagonist, flutamide. Macrophage numbers per testis were reduced by 60% after T-E implantation (Fig. 2B). Macrophage numbers were par-

tially restored after 10 days of hCG treatment, and this restoration was not affected by coadministration of flutamide. The ratio of Leydig cell to macrophage number was 3.38 ± 0.28 in vehicle-treated control animals, and following T-E implantation, this ratio increased to 4.93 ± 0.14 .

Testosterone-estradiol implantation caused a significant reduction in the mean nuclear diameter of testicular macrophages from $5.4 \mu\text{m}$ to $4.9 \mu\text{m}$ (Fig. 3). Administration of hCG alone or in combination with flutamide had no significant effect on macrophage mean nuclear diameter in the testes of these rats (Fig. 3).

Discussion

The data in the present study reinforce our findings in the GnRH-immunized model (Duckett et al, 1997) that maintenance of both Leydig cell and macrophage number in the adult rat testis is primarily dependent upon LH. These data further suggest that, while LH-maintenance of Leydig cell number in the adult is, in part, regulated by androgens, testicular macrophages are regulated by a non-androgenic mechanism that remains to be identified.

Leydig cells and macrophages were readily distinguished in the control testes in $25\text{-}\mu\text{m}$ -thick methacrylate sections when stained with PAS and hematoxylin. In our previous study, we quantified Leydig cells and macrophages in the interstitium of the adult rat testis using the optical disector approach and found this method to be an efficient and unbiased estimator of cell number, with the results obtained being in good agreement with our previous report (Duckett et al, 1997). In thick sections, it was possible to visualize the entire nucleus, greatly facilitating the recognition of these cells by their distinct nuclear morphologies. Vehicle-treated control rats had similar numbers of Leydig cells and macrophages and appeared qualitatively similar to untreated rats as reported in our previous study (Duckett et al, 1997).

Leydig cell regression, as represented by the apparent reduction in nuclear and cytoplasmic volume, was observed in T-E-implanted rats in the present study. This observation is consistent with previous descriptions of Leydig cells in this model (Keeney et al, 1988, 1990; Mendis-Handagama and Ewing, 1990; Mendis-Handagama 1992) and after hypophysectomy (Keeney and Ewing, 1990; Russell et al, 1992; Gaytan et al, 1994) or GnRH immunization (Duckett et al, 1997). However, in the present study, the decline in Leydig cell numbers was considerably greater than previously reported in hypophysectomized or GnRH-suppressed studies, including our own, and in all but two of the previous studies using T-E implantation (Mendis-Handagama and Ewing, 1990; Mendis-Handagama, 1992). The latter study used an un-

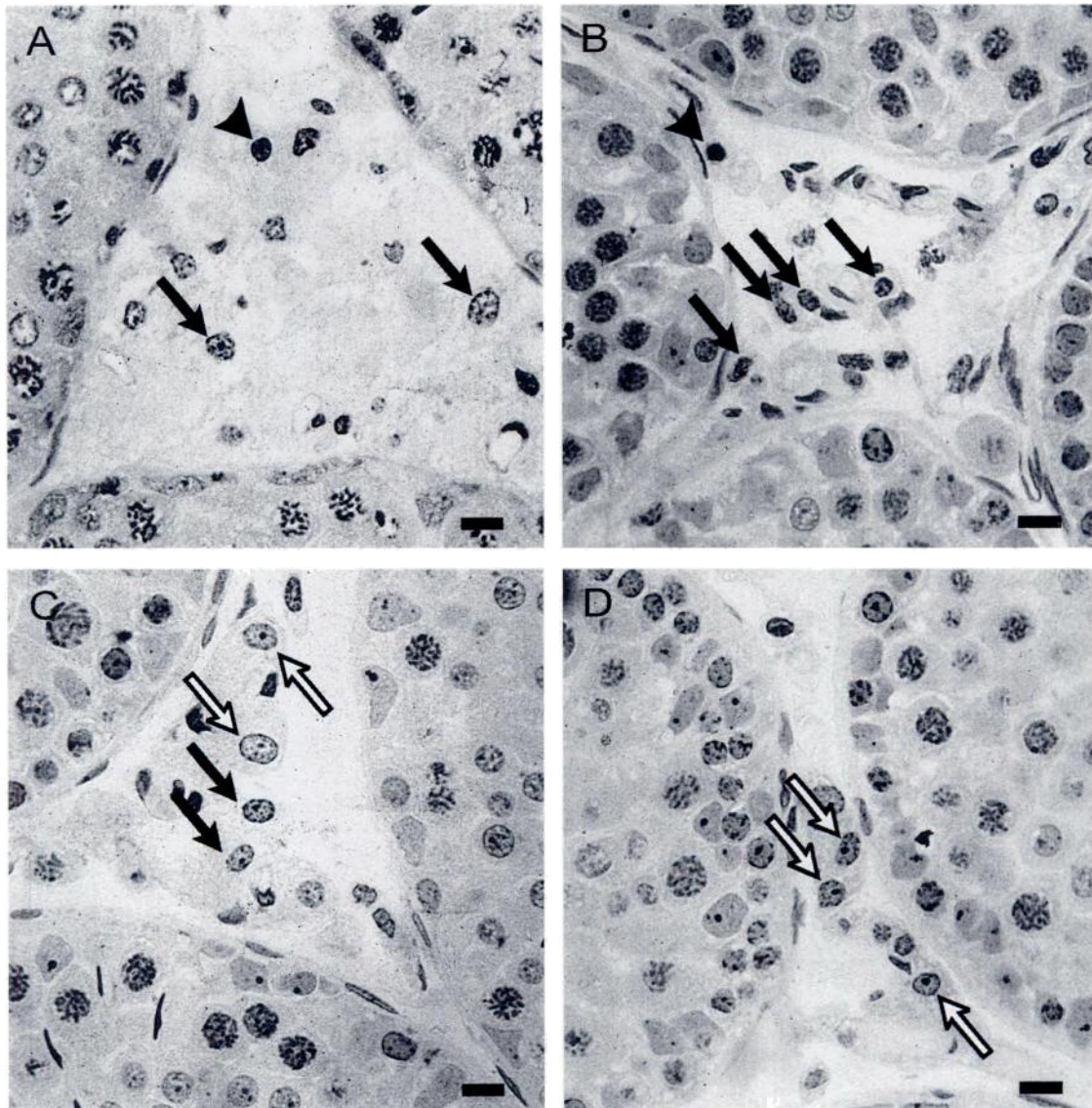


FIG. 1. Light micrograph of the interstitium of the adult rat testis. **(A)** Leydig cells (arrows) identified by their flattened ovoid nuclei, characteristic punctate chromatin, and frequently observed nuclear furrow (not visible in thin sections) and macrophage (arrowhead) in vehicle-treated control animals. **(B)** Regressed Leydig cells (arrows) and macrophage (arrowhead) in animals treated with testosterone-estradiol (T-E) implants for a period of 9 weeks followed by treatment with vehicle for 10 days. **(C)** Adult-type Leydig cells (solid arrows), immature Leydig cells (outline arrows) characterized by a more rounded nucleus with a more prominent single nucleolus and less punctate peripheral heterochromatin, and elongated spermatids in animals treated with T-E implants for a period of 9 weeks followed by human chorionic gonadotropin (hCG) treatment for 10 days. **(D)** Immature Leydig cells (outline arrows) in animals treated with T-E implants for a period of 9 weeks followed by hCG treatment in combination with flutamide for 10 days. Scale bar represents 10 μm .

biased methodology comparable to that employed in the present study.

In the present study, Leydig cells were identified by characteristic nuclear morphologies in PAS and hematoxylin-stained thick sections. These morphological criteria have been well validated in many previous studies (Kerr and Sharpe, 1985a,b; Kerr et al, 1987; Keeney et al, 1988; Hardy et al, 1989; Teerds et al, 1989a,b,c; Keeney and Ewing, 1990; Keeney et al, 1990; Mendis-Handagama and Ewing, 1990; Mendis-Handagama, 1992; Russell et al, 1992; Misro et al, 1992, 1993; Duckett et al, 1997)

and include both adult-type and immature Leydig cells, as well as regressed Leydig cells. However, Leydig cell precursors would not be identified by these criteria. Therefore, it is relevant that a similar decline in Leydig cell numbers in T-E-implanted rats was reported by both Mendis-Handagama and Ewing (1990) and Mendis-Handagama (1992) using a similar morphological approach. By contrast, those studies in which T-E implantation was reported to cause only a small or negligible decline in Leydig cell numbers employed toluidine blue staining in thin sections verified with histochemical localization of

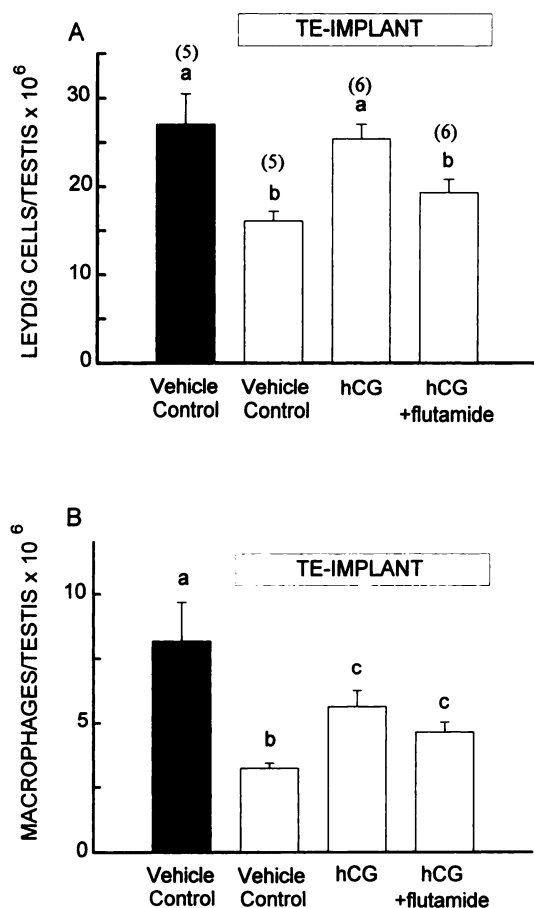


FIG. 2. Effect of human chorionic gonadotropin (hCG) treatment (1.25 IU/kg body weight daily) and hCG treatment in combination with flutamide (100 mg/kg body weight daily) over 10 days on (A) Leydig cell number per testis and (B) macrophage number per testis in testosterone-estradiol (T-E)-implanted adult rats. Values with the same letter superscript are not significantly different ($P > 0.05$).

3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity (Keeney and Ewing, 1990). These latter criteria would presumably also include Leydig cell progenitors or undifferentiated Leydig cell precursors (Keeney and Ewing, 1990). This raises the possibility that the increase in Leydig cell numbers seen after hCG treatment in T-E rats may be due to the maturation of precursors that are not identifiable as Leydig cells by our criteria. The possibility that T-E implants cause both Leydig cell regression and dedifferentiation needs to be considered but is consistent with the observation that both androgen and LH are required for Leydig cell differentiation (Hardy et al, 1990; Misro et al, 1992; Benton et al, 1995).

After hCG administration to T-E-implanted rats, intratesticular concentrations of testosterone rose (Meachem et al, 1997), and Leydig cell number was restored; however, many of these Leydig cells exhibited a nuclear morphology characteristic of immature Leydig cells as described by Kerr and Sharpe (1985a,b) and Kerr et al

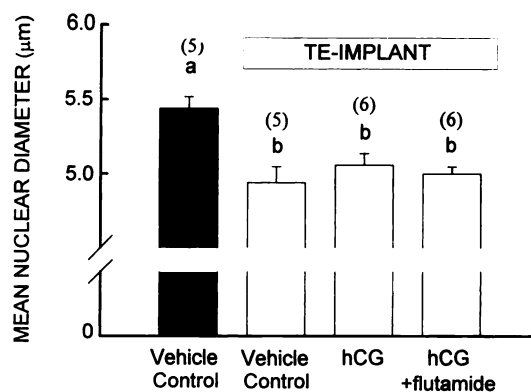


FIG. 3. Effect of human chorionic gonadotropin (hCG) treatment (1.25 IU/kg body weight daily) and hCG treatment in combination with flutamide (100 mg/kg body weight daily) over 10 days on the mean nuclear diameter of testicular macrophages (μm) in testosterone-estradiol (T-E)-implanted adult rats. Values with the same letter superscript are not significantly different ($P > 0.05$).

(1987). When androgen receptors were blocked by flutamide, even in the presence of hCG, there was no significant increase in Leydig cell number even though Leydig cells of both immature and adult-type nuclear morphology were present, supporting the view that LH stimulates Leydig cell proliferation and differentiation via androgen action (Benton et al, 1995). This also is supported by Misro et al (1992) who reported that replacement of testosterone, even in the absence of LH and FSH, in the GnRH-antagonist-treated adult rat restored the number and morphology of the regressed Leydig cells to normal.

Why the decline in differentiated Leydig cells is not so pronounced in the hypophysectomized or GnRH-suppressed studies compared with the T-E-implanted model remains to be determined. The dosage of estradiol and testosterone used in this study act synergistically to suppress testicular activity by feedback inhibition on the hypothalamus and pituitary (Robaire et al, 1979). However, estradiol also has effects on Leydig cells that may alter their morphology in conditions of gonadotropin withdrawal or tubule regression. Leydig cells in the rat have estradiol receptors (Lin et al, 1982), and estradiol inhibits Leydig cell steroidogenic function directly (Kalla et al, 1980). Recent data have also shown that estrogen receptor knockout leads to marked physiological and morphological changes in the mouse testis and infertility (Eddy et al, 1996). The role of estradiol in regulating the differentiated functions of the Leydig cell have received little examination.

Testicular macrophages were reduced in number after T-E implantation, as has been observed after GnRH immunization (Duckett et al, 1997) and hypophysectomy (Gaytan et al, 1994). Replacement of LH by hCG partially restored macrophage numbers. We have proposed that macrophage numbers in the adult testis are dependent

upon Leydig cell number and activity rather than directly upon gonadotropin levels (Wang et al, 1994), and our present data support this hypothesis. Raburn et al (1993) have shown previously that the proliferation of macrophages in the testis interstitium of immature rats treated with Casodex occurs independently of androgens. The data in this study is in support of the proposition that it is the secretion of nonandrogenic products of Leydig cells, or even direct contact (Hutson, 1994), that maintains testicular macrophage numbers, since hCG stimulation of macrophage number was not affected by flutamide in the T-E-treated adult rats in the present study. Although macrophage numbers did not return to vehicle-treated control levels after 10 days of hCG treatment in contrast to the response of Leydig cells, we speculate that macrophages may require a longer time frame for full recovery.

Previous studies in our laboratory strongly indicate that while LH maintains testis macrophage number (Wang et al, 1994), FSH may be responsible for the transition of macrophages to their mature testicular phenotype, including an increase in nuclear size (Duckett et al, 1997). In the present study, the mean nuclear diameter of macrophages was significantly reduced in all T-E-treatment groups compared with vehicle-treated controls, most presumably due to the prolonged fall in FSH concentration. Although FSH levels are acutely restored in T-E-treated animals after hCG administration, the length of time and critical dose may not have been sufficient to induce macrophage recovery in the time frame of the experiment. This is suggested by the slight, although not significant, increase in macrophage nuclear diameter seen in the hCG-treated animals.

As observed in other models of testicular failure (Gaytan et al, 1990; Bergh et al, 1993; Hedger et al, 1995; Duckett et al, 1997), T-E treatment induced the appearance of inflammatory cells in the testicular interstitium. Both mast cells and eosinophils were found in the intertubular tissue as reported and discussed earlier in relation to the GnRH-immunized model (Duckett et al, 1997). As in our previous study, our counting regime did not have the sensitivity required to elucidate the numbers of these cells under the different treatments; however, their absence in the intertubular tissue of vehicle-treated control animals and their presence in all T-E-treatment groups was noted.

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