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17β-Estradiol Inhibition of Leydig Cell Regeneration in the Ethane Dimethylsulfonate-Treated Mature Rat

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ABSTRACT: This study was designed to determine the effects of 17β-estradiol (E2) on Leydig cell development in the rat. Mature (60 to 65 days old) male rats received a single intraperitoneal injection of ethane dimethylsulfonate (EDS, 100 mg/kg body weight); untreated rats served as controls. In one series of experiments, groups of EDS-treated rats also received daily injections of either E2 (25 μg/100 g body weight), human chorionic gonadotropin (hCG, 20 IU/day), a combination of the two, or vehicle only (EDS controls). Animals were killed on days 2, 4, 10, 16, 24, 30, and 36 after EDS treatment. In another series of experiments, groups of EDS-treated rats received daily injections of hCG and E2 during days 0 through 5, 5 through 30, or 16 through 30 after EDS treatment, and were killed on day 30. In both series of experiments, the steroidogenic capacity and hCG binding capacity of the Leydig cells were examined in short-term in vitro incubations using collagenase-dispersed interstitial cells. Testes were also prepared and examined histologically by light and electron microscopy. E2 treatment of animals during the initial 5 days after EDS administration had no effect on the regeneration of interstitial cells and Leydig cells. Treatment with E2 during days 5 to 30 post-EDS blocked the regeneration of Leydig cells and thereby significantly reduced the increase in interstitial cell numbers. Finally, when E2 treatment was delayed until 16 days post-EDS, there was no significant reduction in the regeneration of interstitial or Leydig cells. These data suggest that an important developmental process that is necessary for Leydig cell regeneration occurs between days 5 and 16 post-EDS. Furthermore, this process appears to be sensitive to the inhibitory action of E2. The results of this study implicate the Leydig precursor cell as an estrogen target cell. These results are the first to demonstrate that daily administration of E2 blocked the reappearance of Leydig cells in the EDS-treated rat.

Key words: estradiol inhibition, precursor cells, ethane dimethylsulfonate (EDS), proliferation, differentiation.

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In the fetal rat testis, the luteinizing hormone (LH) receptor and a stimulatory guanine nucleotide protein (G_s) become functional at 14.5 days of gestation (Warren, 1989), and Leydig cells are histologically identifiable on day 15 of gestation (Roosen-Runge and Anderson, 1959). Leydig cell numbers increase rapidly through parturition and reportedly decrease in the initial 10 days after birth (Lording and de Kretser, 1972). Recently, Kerr and Knell (1988) demonstrated that the fetal population of Leydig cells persists in the adult testis and does not undergo postnatal degeneration. During the third and fourth postnatal weeks, a second "adult" population begins to develop (Roosen-Runge and Anderson, 1959; Lording and de Kretser, 1972) and increases in number through days 50 to 60, after which it remains constant. Leydig cells in both the rat and human are believed to be derived from mesenchymal precursor cells (Mancini et al, 1963), which are characterized by a high level of proliferation in the first days after birth (Niemi and Ikonen, 1963). Whereas little mitotic activity is observed in the Leydig cells of the mature rat (Mori and Christensen, 1980), Leydig cells of the immature rat are capable of cell division (Roosen-Runge and Anderson, 1959; Chemes et al, 1976). More recently, Hardy et al (1989) presented evidence suggesting that mesenchymal cells give rise to Leydig cells through day 28. Beyond day 28, newly generated Leydig cells are capable of cell division, thereby generating additional Leydig cells. Thus, Leydig cell development is viewed as a process of precursor cell division followed by cellular differentiation and maturation to yield mature Leydig cells.

In recent years, the ethane dimethylsulfonate (EDS)-treated rat has become useful as a model for studying Leydig cell development and function. EDS is an alkylating agent that has a cytotoxic effect on Leydig cells (Kerr et al, 1985; Rommerts et al, 1985; Morris et al, 1986). Although its specific site of action is unknown, within 24 hours of a single intraperitoneal injection of EDS, Leydig cell death occurs. Within 2 to 3 days after treatment, all Leydig cells are eliminated by the phagocytic activity of invading macrophages (Kerr et al, 1985; Molenaar et al, 1985; Kerr et al, 1988). An intriguing aspect of this model is the subsequent regeneration of Leydig cells that occurs during weeks 3

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through 7 after EDS treatment. Small Leydig cells, presumably derived from fibroblastic precursor cells (Edwards et al, 1987; Kerr et al, 1987; Teerds et al, 1988), are present around day 21, and Leydig cells appear to be normal by days 40 through 49 (Morris et al, 1986; Jackson et al, 1986a; Jackson et al, 1986b). The final stage of precursor cell differentiation is believed to be rapid, since Leydig cell numbers increase markedly between days 21 and 28 (Edwards et al, 1987). Recent evidence indicates that Leydig cell regeneration in the EDS-treated rat occurs in at least two waves of proliferation. The first wave is characterized by precursor cell (mesenchymal) division, and the second wave involves division of newly formed Leydig cells (Teerds et al, 1990; Myers and Abney, 1991). These results support the observations of Hardy et al (1989) in the pubertal rat. The cellular events that occur during this period in the EDS-treated rat appear to be similar to those of normal Leydig cell ontogeny in the prepubertal and pubertal rat.

With the discovery that the testis concentrates estradiol ([E_2], Stumpf, 1969), and subsequent reports that an estrogen receptor is present in the testis (Brinkmann et al, 1972; Kato et al, 1974; Abney, 1976), interest in the possible direct effects of estrogens in the testis grew. Evidence suggests that E_2 may be involved in the regulation of Leydig cell development. Studies demonstrating the absence of mature Leydig cells in the testes of 60-day-old rats that had received a single injection of E_2 at 5 days of age (Dhar and Setty, 1976) suggest that Leydig cell development is sensitive to estrogens. In subsequent studies, ^{3}H-thymidine incorporation *in vivo* (Saez et al, 1978) and *in vitro* (Abney and Carswell, 1986) by interstitial cells from mature rats was shown to be stimulated by administration of human chorionic gonadotropin (hCG). In each study, E_2 treatment inhibited ^{3}H-thymidine incorporation of both control (nontreated) and hCG-treated mature rats. Both studies indicate that DNA synthesis in interstitial cells is enhanced by gonadotropins, and that this stimulatory effect is blocked by the inhibitory action of E_2.

We investigated the possible inhibitory role of E_2 in the process of Leydig cell regeneration in the EDS-treated mature rat. The data presented here suggest that E_2 may be involved as either a paracrine or autocrine factor in the modulation of Leydig cell ontogeny.

Materials and Methods

Animals and Treatment

Mature (60 to 65 days old) Sprague Dawley rats, purchased from Harlan Industries (Indianapolis, IN), were maintained in a 12-hour light: 12-hour dark environment with *ad libitum* access to water and rat chow. EDS was synthesized by the procedure of Jackson and Jackson (1984) as previously described (Myers and Abney, 1989), dissolved in dimethyl sulfoxide and water (1:3, v/v), and administered as a single intraperitoneal injection (100 mg EDS/kg body weight). In one series of experiments, animals received daily subcutaneous injections of either vehicle, E_2 (25 μg/100 g body weight), hCG (20 IU/rat), or a combination of E_2 and hCG. Animals were killed on days 2, 4, 10, 16, 24, 30, and 36 after EDS treatment, and their testes were removed. A second series of experiments was conducted to determine the period when Leydig cell regeneration is sensitive to the suppressive actions of E_2. Groups of EDS-treated rats received daily injections of hCG and E_2 during days 0 through 5, 5 through 30, or 16 through 30 after EDS treatment, and were killed on day 30. Age-matched, vehicle-treated controls were also killed at each of the above days. Each experiment, using two animals per group, was conducted at least twice.

Hormones, Buffers, and Solutions

E_2 was obtained from Steraloids, Inc. (Wilton, NH) and hCG (CR-121) was supplied by the Population Research Branch of the National Institute of Child Health and Human Development. hCG was iodinated by a modified procedure (Leidenberger and Reichert, 1972) using chloramine T; specific activity was 35 to 46 μCi/μg, equivalent to 60,000 cpm/ng. Collagenase (180 to 210 U/mg) was purchased from Cooper Biomedical (Malvern, PA); hCG and dibutyryl cyclic adenosine monophosphate (dbcAMP), used for stimulation of steroidogenesis in vitro, were obtained from Rugby Laboratories (Rockville Center, NY) and Sigma Chemical Co. (St. Louis, MO), respectively. Incubations for androgen production and hCG binding were performed *in vitro*, as described previously (Keel and Abney, 1982; Myers and Abney, 1990), in a 1:1 mixture of Dulbecco's Modified Eagles Medium and Ham's F-12 (DMEM/F12), both obtained from Gibco (Grand Island, NY), and supplemented with sodium bicarbonate 1.2 g/L and 15 mmol/L Hepes (Gibco). Radioimmunoassays (RIA) were performed in phosphate-buffered saline containing 0.1% gelatin (Keel and Abney, 1982; Myers and Abney, 1990), using a charcoal (Amend Co., Irvington, NJ) and dextran (ICN, Cleveland, OH) suspension to separate bound and free steroids.

Preparation of Interstitial Cells

One testis from each animal was dispersed in 2 ml DMEM/F12 with 0.5 mg collagenase (Myers and Abney, 1990). Dispersed interstitial cells were separated from the tubular sediment by filtration through surgical gauze, centrifuged twice at 800g for 10 minutes in DMEM/F12 to remove the collagenase, and then diluted to a final concentration of 1×10^6 cells/ml in DMEM/F12. These cell preparations represented the recoverable interstitial cells that were obtained from the collagenase-dispersed testes. This cell preparation is considered more likely to contain the precursor cells than a purified Leydig cell preparation. Use of these cells will therefore permit the detection of Leydig cell markers that might be exhibited by precursor cells.

In Vitro Incubations and Hormone Assays

Interstitial cells were incubated at a concentration of 1×10^6 cells/ml for determination of steroidogenic capacity (Keel and Abney, 1982) in the presence or absence of 10 mIU hCG or 1.0 mmol/L dbcAMP in a final volume of 1 ml. Steroid levels were

determined by RIA. Antiserum to testosterone (x-181) was obtained from Radioassay Systems Laboratories, Inc. (Carson, CA). Due to the high specificity of the antiserum used in these studies (Keel and Abney, 1985), assays were performed directly on incubation media. LH receptor levels were determined by the addition of iodine-125-labeled hCG at a concentration of 4.0×10^5 cpm/10^6 cells in the presence or absence of a 1,000-fold excess of unlabeled hCG, as previously described (Myers and Abney, 1988). After a 3-hour incubation, cells were rinsed and centrifuged twice in cold DMEM/F12, and the bound radioactivity was determined.

Histologic and Morphologic Analyses

For histologic analysis using a light microscope, testicular tissue was prepared as described previously (Myers and Abney, 1990). In brief, tissue was fixed in Bouin's solution, embedded in paraffin, and cut into 6- to 8-μm-thick sections (Christensen and Peacock, 1980). Sections were stained with hematoxylin–eosin, overlaid with coverslips, and observed with a Leitz microscope at a magnification of ×400. Triangular interstitial areas surrounded by three cross-sectioned seminiferous tubules were chosen for counting, as previously reported (Bergh, 1982). A minimum of 160 interstitial spaces was counted for each experimental group. This involved counting an average of 4 interstitial spaces in each of 10 fields per slide from a minimum of 4 slides per group. Leydig cells were identified by the presence of a large oval nucleus with peripheral heterochromatin and a prominent nucleolus, in addition to an abundant eosinophilic cytoplasm (Lording and de Kretser, 1972; Laws et al, 1985; Edwards et al, 1987).

Morphologic analysis using the electron microscope was performed as follows: testicular tissue was cut into small pieces and fixed for 2 hours by the method of Ito and Karnovsky (1968), and then placed in Sorenson's phosphate buffer overnight. Tissue was post-fixed in 1% osmium tetroxide for 2 hours, dehydrated through graded changes in ethanol, (70% to 100%) and transferred to propylene oxide. Tissue was embedded in an Epon-araldite mixture and cut into 80- to 100-nm-thick sections using a DuPont diamond knife on a Sorvall ultramicrotome (DuPont Instruments, Wilmington, DE). Sections were stained with 2% uranyl acetate for 60 minutes and lead citrate for 5 minutes. Sections were viewed on a Phillips 400 electron microscope (Eindhoven, The Netherlands).

Statistics

Statistical differences among experimental groups were analyzed by one-way analysis of variance and student's t test. Results are expressed as the means ± SEM, and significant differences are noted in the results.

Results

Testicular Weight

The first parameter measured was testicular weight, as shown in Figure 1. The value for the control group, 1.79 ± 0.07 g/testis, is the mean ± SEM of all the age-matched, vehicle-treated controls, among which no significant differences were noted. Testis weight in the EDS/hCG-treated group dropped to 0.99 ± 0.08 g on day 16 after EDS treatment, and returned to control levels by day 36. It was noted that daily hCG treatment had no influence on the pattern of testicular weight loss and recovery in comparison to information reported for EDS treatment alone (Myers and Abney, 1990). Treatment with EDS and hCG plus E_2 (EDS/hCG/E_2) resulted in a significantly greater decrease ($P \leq 0.05$) in testicular weight at days 10 and 16 in comparison to the EDS/hCG treatment. Furthermore, treatment with E_2 prevented the recovery of testicular weight, which occurred in the EDS/hCG-treated group between days 16 and 36, as depicted in Figure 1. It should be noted that the testicular weights of rats treated with hCG/E_2 were not different from those treated with E_2 alone (data not shown).

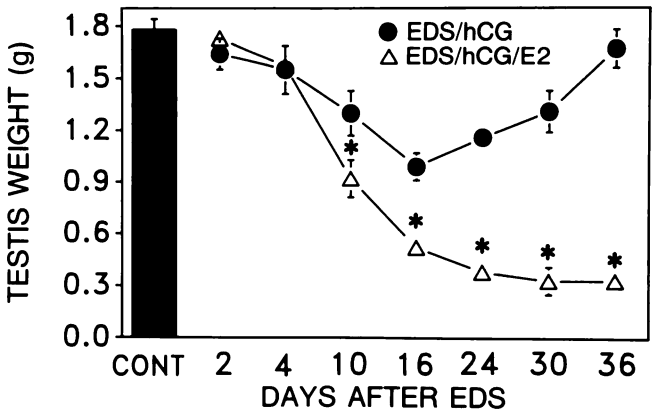


FIG. 1. The effect of E_2 on testis weight in the EDS-treated mature rat. Animals received a single injection of EDS (100 mg/kg body weight) followed by daily treatment with either hCG (20 IU/day) or hCG and E2 (25 μg/100 g body weight). Control animals were not treated. The control value is the mean ± SEM of 14 to 20 age-matched animals. For each of the experimental groups, the values are presented as the mean ± SEM of six to eight testes. *$P < 0.05$ *versus* LH treatment.

Testosterone Production In Vitro

Interstitial cells isolated from age-matched controls produced basal testosterone levels of 0.90 ± 0.07 ng/10^6 interstitial cells/3 hours *in vitro*. Addition of 10 mIU hCG resulted in a 4.6-fold stimulation, yielding 4.2 ± 1.0 ng testosterone/10^6 cells/3 hours. Addition of 1.0 mmol/L db-cAMP produced a maximum stimulation of testosterone production (data not shown) equivalent to that obtained with 10 mIU hCG. As is evident from the data in Table 1, interstitial cells from all EDS-treated groups did not produce detectable levels of hCG-stimulated testosterone during days 2 through 10. Basal and hCG-enhanced steroidogenesis was first measurable in the EDS and EDS/hCG-treated groups at day 16, and rose to values that were equivalent to the controls on days 24 through 36. Daily hCG treatment *in vivo* had a negligible effect on testosterone production in the EDS/hCG-treated group through the course of these studies.

Table 1. *The effect of estradiol treatment* in vivo *on testosterone production by interstitial cells isolated from EDS-treated rats*

Days after EDS	Testosterone (ng/10^6 cells/3 hr)			
	EDS	EDS/E_2	EDS/hCG	EDS/hCG/E_2
2	—*	—	—	—
4	—	—	—	—
10	—	—	—	—
16	0.65 ± 0.10	—	1.2 ± 0.6	—
24	3.8 ± 0.85	—	4.0 ± 0.7	—
30	6.2 ± 0.5	0.25 ± 0.15†	8.5 ± 2.0	0.75 ± 0.1‡
36	5.2 ± 1.3	0.32 ± 0.19†	ND	ND

Testosterone production in the presence of 10 mIU hCG. The values are presented as the mean ± SEM of two experiments, each utilizing pooled interstitial cells from four rats per group. Control values = 4.2 ± 1.0 ng/10^6 cells/3 hr. ND = not done.

* Nondetectable levels of testosterone.

† Significance ($P < 0.05$) *versus* appropriate EDS values.

‡ Significance ($P < 0.05$) *versus* appropriate EDS/hCG values.

Treatment with E_2, either alone or with hCG, prevented recovery of the steroidogenic capacity compared to groups not treated with E_2. Low levels of hCG-stimulated testosterone production were detectable at days 30 and 36 in the E_2-treated groups.

Gonadotropin Binding In Vitro

The hCG binding capacity of isolated interstitial cells was examined after EDS injection and the various hormonal treatments, as summarized in Table 2. The mean hCG binding capacity of the age-matched controls was 65 ± 15 pg I-125–labeled hCG/10^6 cells, representing a value of 100% ± 24%. EDS administration resulted in the complete loss of hCG binding capacity by day 2. Treatment of animals with hCG delayed the loss of gonadotropin receptors on days 2 and 4. Nevertheless, by day 10, the loss of hCG binding was comparable to that in the animals that did not receive hCG treatment. Recovery of hCG binding was not altered by daily hCG treatment, and, similar to the EDS-treated group, binding in the EDS/hCG-treated group was equivalent to control values by day 36. As shown in Table 2, E_2 treatment had no effect on the dramatic loss of hCG binding capacity that occurred during days 2 through 16. In comparison to the EDS-treated group, the recovery of hCG binding capacity was reduced significantly ($P \leq 0.05$) on days 24 through 36 in the EDS/E_2-treated group. In the EDS/hCG/E_2-treated group, the recovery of gonadotropin binding capacity was likewise inhibited, and reached only 20% of the control level by days 30 and 36.

Histologic Analysis of Interstitial Cells

Histologic studies were performed to determine the presence or absence of Leydig cells within the testis of the EDS-treated rat following daily injections with E_2. As shown in Figure 2A, Leydig cell clusters were observed within the interstitial spaces of EDS/hCG-treated rats 30 days after EDS treatment. The testes of EDS/hCG/E_2-treated rats did not contain identifiable Leydig cells 30 days after EDS treatment (Fig 2B). The interstitium of these testes contained elongate cells with scanty cytoplasm and nuclei that stained darkly with hematoxylin. In addition, the tubules of these animals remained atrophic. Studies conducted with electron microscopy verified the lack of Leydig cells within the interstitium of rats treated in this manner (EDS/hCG/E_2) 30 days after EDS.

The numbers of total interstitial cells and Leydig cells,

Table 2. *The effect of estradiol treatment* in vivo *on hCG binding capacity of interstitial cells isolated from EDS-treated rats*

Days after EDS	hCG binding (% of control)			
	EDS	EDS/E_2	EDS/hCG	EDS/hCG/E_2
2	14 ± 10	13 ± 8.0	40 ± 12	24 ± 10
4	8.0 ± 8.0	7.5 ± 2.8	22 ± 8.0	14 ± 5.0
10	13 ± 3.0	8.0 ± 8.0	3.7 ± 0.9	2.7 ± 0.8
16	8.1 ± 4.0	4.0 ± 1.5	6.6 ± 2.0	3.4 ± 0.6
24	26 ± 4.0	7.0 ± 2.0*	21 ± 6.0	3.4 ± 0.5†
30	62 ± 11	21 ± 6.0*	80 ± 10	19 ± 5.0†
36	90 ± 3	32 ± 5.0*	ND	ND

Rats received a single injection of EDS (100 mg/kg body weight) followed by daily treatment with E_2 (25 μg · 100 g body weight^{-1} · day^{-1}) and/or hCG (20 IU/day). The values are expressed as percent of control and presented as the mean ± SEM of two to three experiments, each utilizing pooled interstitial cells from four rats per group. Control = 100 ± 24%. ND = not done.

* Significance ($P < 0.05$) *versus* appropriate EDS values.

† Significance ($P < 0.05$) *versus* appropriate EDS/hCG values.

expressed per triangular interstitial space, were quantified histologically. Age-matched controls exhibited 14.1 ± 1.1 total interstitial cells and 6.0 ± 1.07 Leydig cells per interstitial space, as depicted in Figure 3. As expected, treatment with hCG/E_2 did not affect the EDS-induced decline in interstitial cell numbers. However, as a result of E_2 administration, interstitial cell numbers did not exhibit an increase after day 16, and were decreased 30% to 35% on days 24, 30, and 36 after EDS, compared to animals that received hCG only (Fig 3A). Leydig cell numbers declined immediately after EDS administration in both the hCG and hCG/E_2-treated groups. In a pattern similar to that observed previously in rats receiving EDS only (Myers and Abney, 1990), Leydig cell numbers increased rapidly after day 16 in the hCG-treated rats, and were not different from control values by day 24 after treatment. In contrast, few Leydig cells were observed during the regenerative phase (days 24 through 36) in rats treated with hCG/E_2 (Fig 3B).

Temporal Nature of E_2 Inhibition

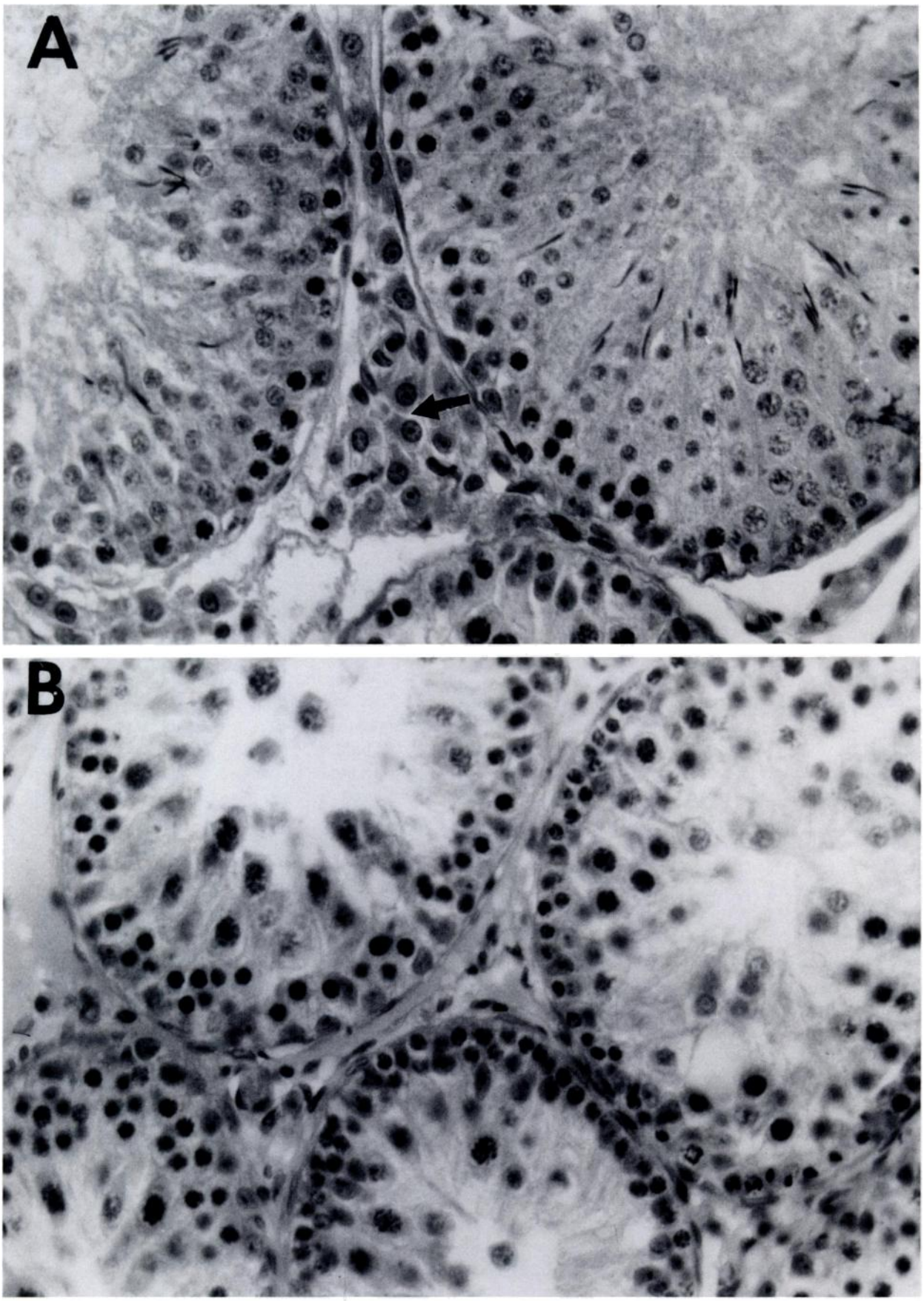
The second series of experiments was conducted to determine the period when Leydig cell regeneration is sensitive to the suppressive actions of estrogens. EDS-treated rats received hCG (controls) or hCG/E_2 for various periods. Daily treatments with hCG or hCG/E_2 were conducted as follows: days 0 through 5 and then discontinued (group A), days 5 through 30 (group B), and days 16 through 30 (group C) after EDS administration. All animals were killed on day 30, and the numbers of interstitial and Leydig cells present after these periods of E_2 treatment were quantified histologically.

As shown in Figure 4A, treatment of rats with E_2 during the initial 5 days after EDS treatment did not affect the number of interstitial cells or Leydig cells per interstitial space observed on day 30. Treatment with hCG/E_2 from 5 to 30 days post-EDS resulted in a decrease from the control value of 13.1 ± 1 to 8.5 ± 0.6 interstitial cells per space. In addition, a dramatic decrease in Leydig cells was observed in this group (Fig 4B). The reductions in interstitial and Leydig cell numbers were significant compared to the numbers of cells in animals treated with hCG only. When hCG/E_2 treatment was begun 16 days after EDS, no significant decrease in total interstitial cell numbers was observed on day 30. Likewise, the number of visible Leydig cells was not significantly reduced from a control value of 4.0 ± 0.6 cells/space. The results from this series of experiments may be summarized as follows: treatment with E_2 during the initial 5 days after EDS had no effect on the regeneration of total interstitial cells and Leydig cells. Treatment with E_2 during days 5 through 30 after EDS blocked the regeneration of Leydig cells and significantly reduced the number of total interstitial cells. Finally, when E_2 treatment was delayed until 16 days after EDS, there was no significant reduction in the number of total interstitial or Leydig cells.

Discussion

The rapid disappearance of Leydig cells by day 2 post-EDS treatment and the regeneration of Leydig cells, first observed around day 21 after EDS treatment, have been well established by investigators in this and other laboratories (Myers and Abney, 1990; Kerr et al, 1985; Molenaar et al, 1985; Jackson et al, 1986a; Jackson et al, 1986b; Morris et al, 1986; Edwards et al, 1987; Kerr et al, 1987; Kerr et al, 1988; Teerds et al, 1988). The data presented here are the first to demonstrate that the process of Leydig cell regeneration is inhibited by estrogens. Previous studies (Molenaar et al, 1986) demonstrated a lack of Leydig cells in the EDS-treated rat in the absence of LH. The final stages of differentiation are reported to be particularly dependent on LH (Teerds et al, 1989a). For this reason, hCG was administered simultaneously with E_2 in the current study to insure that the effects of E_2 occurred independently of pituitary function. A single high-dose injection of hCG (100 IU or more) has been shown to disrupt spermatogenesis (van Vliet et al, 1988; Kerr and Sharpe, 1989). There is evidence, however, that lower doses (eg, 12.5 IU) do not impair the spermatogenic process (Bergh et al, 1986). We used 20 IU hCG, a relatively low dose of gonadotropin, and daily administration did not alter the patterns of degeneration or regeneration of interstitial cells in the EDS-treated animal.

hCG binding capacity provided an excellent marker of Leydig cell regeneration because Leydig cells are the only cell type within the testis that contain the LH receptor (Mancini et al, 1967). Although LH is required for Leydig cell regeneration in the EDS-treated rat, all evidence suggests that hCG binding is detectable only after identifiable Leydig cells are present. Gonadotropin binding by precursor cells has yet to be demonstrated. In fact, there currently are no morphologic or biochemical markers that can be attributed to the precursor cell. Thus, there is no empirical evidence for identification of the precursor cell. The low level of hCG binding observed in the EDS/E_2-treated rat at days 24, 30, and 36 after treatment strongly suggests estrogen inhibition of Leydig cell development. Furthermore, the low level of hCG binding observed in the EDS/hCG/E_2-treated rat at 24 and 30 days post-EDS indicates that the inhibitory action was mediated directly in the testis, independent of the pituitary. An interesting observation made in this study was the increased hCG binding capacity observed at days 2 and 4 after EDS in the EDS/hCG-treated rat, compared to the EDS-only-treated rat. This suggests a protective influence of gonadotropin on the interstitium of the testis, as proposed in an earlier study (Jackson and Jackson, 1984). It should be noted, however, that gonadotropin did not maintain the hCG binding capacity after day 4 post-EDS in these studies; thus, any protective influence was transient in nature. Several investigators have suggested that estrogen treatment of rats (Saez et al, 1978) and humans (Huhtaniemi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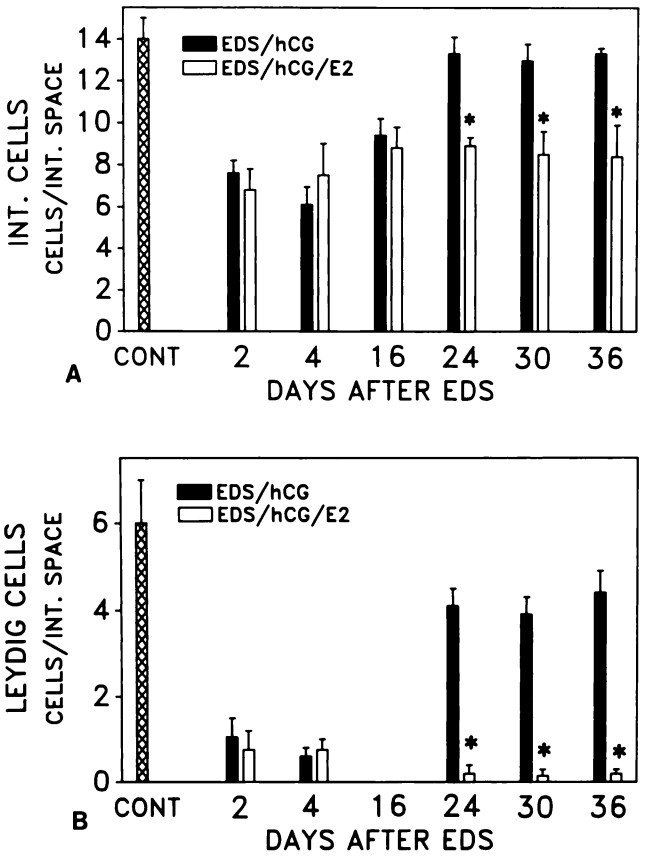

FIG. 3. The effect of E_2 on the numbers of (panel A) total interstitial cells and (panel B) Leydig cells in the EDS-treated mature rat. Animals received a single injection of EDS (100 mg/kg body weight), were treated daily with hCG (20 IU/day) or hCG and E_2 (25 μg/100 g body weight), and were killed on the days indicated. Control animals were not treated. Cells were quantified per interstitial space. Values are presented as the mean ± SEM of the average counts obtained from each of two to four animals. *P < 0.05 *versus* appropriate age-matched group treated with hCG only.

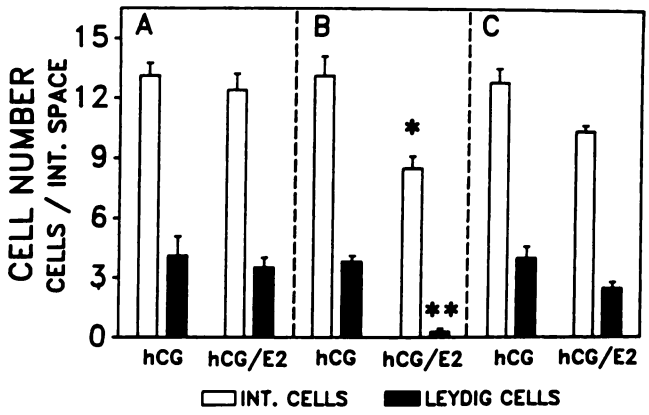

FIG. 4. The effect of E_2, administered for various periods after EDS treatment, on Leydig cell development in the mature rat. Animals received a single injection of EDS (100 mg/kg body weight) and were treated daily with hCG (20 IU/day) or hCG and E_2 (25 μg/100 g body weight) for (A) 0 to 5 days after EDS, (B) 5 to 30 days after EDS, and (C) 16 to 30 days after EDS. All rats were killed 30 days after EDS and cells were quantified. The values for both total interstitial cells (□) and Leydig cells (■) are presented as the mean ± SEM of the average counts obtained from each of 2 to 4 animals. *P < 0.05 *versus* appropriate hCG-treated group value.

et al, 1980) may decrease the LH binding capacity of the testis. The possibility that E_2 did not inhibit Leydig cell regeneration, but instead blocked the reappearance of the LH receptor in the EDS-treated rat, was considered. This seems unlikely based on the histologic studies that demonstrate the absence of visible Leydig cells at 24, 30, and 36 days post-EDS in the hCG/E_2-treated rats.

It is interesting to note that although histologically identifiable Leydig cells were absent from the interstitium at days 30 and 36 post-EDS in EDS/E_2-treated rats, the hCG binding capacity of isolated interstitial cells from these rats was 20% and 33% of the control value, respectively. One possible explanation is that some of the interstitial cells did not display the morphologic characteristics of Leydig cells, yet they possessed LH/hCG receptors. Whether these cells could represent a Leydig precursor cell arrested by E_2 treatment at some point in the process of differentiation is not currently known. These results must be interpreted with caution for several reasons. First, collagenase-dispersed interstitial cells contain a substantial number of germ cells. Because E_2 treatment, in the presence or absence of hCG, resulted in a drastic reduction in the number of germ cells (based on histologic observation), collagenase-dispersed interstitial cell preparations from E_2-treated rats contained a higher proportion of true interstitial cells, that is, a lower number of germ cells as compared to cell preparations from rats treated with EDS only. Furthermore, it is not possible to determine if this increase in hCG binding was the result of increased Leydig cell numbers or increased binding per cell.

Histologic quantification of total interstitial cells and Leydig cells provided valuable insight into the inhibitory effect of E_2 on Leydig cell development. As pointed out by Christensen and Peacock (1980), there are only two ways in which the numerical density of Leydig cells can change: 1) the total number of Leydig cells per testis can vary; or 2) the testis size can vary such that the same number of Leydig cells would be contained in a different tissue volume. In the current study, triangular interstitial spaces were chosen to provide an easily identifiable histologic structure within the testis as a reference for cell counts. E_2 did not affect de-

FIG. 2. Light micrograph of the testis from a rat 30 days after EDS treatment. Panel A: Animals were treated daily with hCG (20 IU/day). Leydig cells (arrow) are present in the interstitium (×1400). Panel B: Animals were treated daily with hCG (20 IU/day) and E_2 (25 μg/100 g body weight). The interstitium is devoid of Leydig cells and degenerative tubules are present (×1400).

generation of Leydig cells in the EDS-treated rat. As mentioned above, the number of interstitial cells in the hCG-treated rat increased rapidly between 16 and 24 days after EDS treatment, and this increase appears to be correlated with the rapid appearance of identifiable Leydig cells at 24 days after EDS. The testis of the E_2-treated rat did not display this increase in total interstitial cells at 24 to 36 days after EDS, and Leydig cells also were not present during this period. These results suggest that E_2 specifically blocked the rapid phase of Leydig cell development that is initiated between days 16 and 24 after treatment. The lack of Leydig cells at this time, together with decreased total interstitial cell number, suggest that the estrogenic inhibition of Leydig cell development is mediated primarily via decreased cell proliferation, rather than inhibition of the differentiation of cells already present within the interstitium. The possibility that precursor cells develop to a certain stage and then undergo estrogen-induced cell death cannot be excluded at this time.

The continuous decline in testis weight observed in the EDS/hCG/E_2-treated rat was probably due to a degeneration of the germinal epithelium in these animals. This was attributable, in part, to the lack of testosterone, which is required for normal maintenance of the germinal epithelium. The lack of testosterone production by isolated interstitial cells from the EDS/hCG/E_2-treated rat does not in itself provide adequate proof for the absence of Leydig cells in this model. It is possible, for example, that Leydig cell regeneration occurred, yet androgen production was inhibited by the exogenously administered E_2, as previously reported (Hsueh et al, 1978; Melner and Abney, 1980; Nozu et al, 1981; Bendeck and Pomerantz, 1984; Freeman, 1985). It is our hypothesis that E_2 blocked the process of Leydig cell regeneration. Support for this hypothesis is provided by the data that demonstrate that the post-EDS decrease in hCG binding capacity was not restored in the EDS/E_2-treated rats, and by histologic evidence that shows that identifiable Leydig cells were absent in EDS/E_2-treated rats.

Numerous cell–cell interactions involving endocrine or paracrine factors regulate testicular development (Sharpe et al, 1990). It is probable, therefore, that Leydig cell regeneration in the EDS-treated animal is partially controlled by factors from the seminiferous tubule. There is evidence that post-EDS regeneration of Leydig cells is more rapid in cryptorchid testes that exhibit damaged tubules, in comparison to control testes (O'Leary et al, 1986). One possible explanation for the data in the current study is that E_2 acted on the seminiferous epithelium and consequently blocked the normal production or secretion of a tubular paracrine factor that is necessary for Leydig cell regeneration. It is also interesting to note that E_2 administration following EDS treatment was recently reported to exert a synergistic cytotoxic effect on the seminiferous epithelium (Sprando et al, 1990).

The lack of an estrogenic effect on the subsequent appearance of Leydig cells in rats that were treated from 0 to 5 days after EDS suggests that cells present during days 0 to 5 are insensitive to estrogens, or that E_2 inhibition occurred and that recovery was complete by day 30 after EDS. It is also conceivable that events related to Leydig cell development are not initiated during this early time period, as suggested by Teerds et al (1989a, 1989b). The complete inhibition of Leydig cell development that occurred when rats were treated with hCG/E_2 between days 5 and 30 after EDS demonstrates the presence of E_2 sensitivity during this period. E_2 treatment between days 16 and 30 after EDS, a time of rapid appearance of Leydig cells, did not suppress the number of interstitial cells or Leydig cells in comparison to the appropriate controls (rats treated with only hCG during the same period). Therefore, E_2 treatment after day 5 and before day 16 post-EDS was sufficient to block the reappearance of Leydig cells. These findings indicate that an event, or series of events, sensitive to the inhibitory action of E_2 and necessary for the development of Leydig cells, occurs between 5 and 16 days after EDS. Further studies are required to elucidate more precisely the onset of estrogen sensitivity.

The Leydig cell is unusual as an estrogen target cell, in that it also possesses a gonadotropin-sensitive aromatase and actively produces E_2 (Canick et al, 1979; Valladares and Payne, 1981). This synthesis, in addition to the selective transfer of estrogens across the pampiniform plexus to the spermatic artery (Amann and Ganjam, 1976), suggest that high concentrations of E_2 are normally present in the testis. In fact, E_2 concentrations in spermatic venous blood were shown to be 10- to 50-fold higher than those in peripheral blood in several species (Kelch et al, 1972). When these parameters are considered, the physiologic concentrations of E_2 within the testis may be sufficient to exert a direct, autocrine-mediated effect on Leydig cells. In this context, E_2 was most effective in blocking Leydig cell regeneration when administered between days 5 and 16, a time when histologically identifiable Leydig cells are absent. This suggests that the Leydig precursor cell is probably the site of the E_2 inhibitory action. It has been postulated that the differentiated mature Leydig cell may produce a substance that inhibits precursor cell proliferation (Teerds et al, 1989b). Perhaps E_2 acts within the testis as a paracrine regulator in controlling precursor cell development.

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