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Pituitary Control of Proliferation and Differentiation of Leydig Cells and Their Putative Precursors in Immature Hypophysectomized Rat Testis

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ABSTRACT: The objective of this study was to determine the effects of pituitary hormones (luteinizing hormone [LH], follicle-stimulating hormone [FSH], growth hormone [GH], and prolactin [PRL]) on interstitial cell proliferation and differentiation in the testis of immature hypophysectomized rats. Macrophages, Leydig cells, precursor mesenchymal cells, endothelial lymphatic cells, and myoid cells were studied. Our experimental approach was aimed at determining whether changes in a cellular subpopulation observed after pituitary hormone treatments were the result of division of existing cells in the population, of differentiation of interstitial precursor cells, or both. In this context, it must be stressed that our data reflected the effects of hormones to prevent the decline of cells due to hypophysectomy rather than their recovery. Macrophage proliferation was taken into account because macrophages closely resemble Leydig cells and are known to proliferate after hormonal treatment. A double-labeling procedure (acid phosphatase and anti-bromodeoxyuridine [anti-BUdR]) revealed that LH, FSH, and PRL increased the number of testicular macrophages 105-, 104-, and 103-fold, respectively, in hypophysectomized rats compared to hypophysectomized control animals. BUdR incorporation in testicular macrophages was greater after PRL treatment than after LH and FSH supplementation. In contrast, we were unable to demonstrate any effect of rat GH on the macrophage population. Light microscopic analysis of plastic embedded sections of treated rat testis revealed that LH increased the numbers of Leydig, precursor mesenchymal, and myoid cells 6-, 4-, and 1.3-fold, respectively. LH also stimulated BUdR incorporation into all interstitial cell types. PRL administration increased both the number of Leydig and precursor mesenchymal cells (each 3-fold) but decreased the number of endothelial lymphatic cells (1.5-fold) when compared to the control animals. In contrast, FSH did not increase the number and proliferation of Leydig cells but exerted a slight proliferative effect on the other interstitial cell populations. In GH-treated rats, the number of precursor mesenchymal cells increased two fold above the control rats. GH also exerted slight proliferative effects on both precursor mesenchymal and myoid cells. Immunohistochemical studies of steroidogenic enzymes in the testicular interstitium of treated rats demonstrated the presence of steroidogenic enzymes, not only in Leydig and precursor mesenchymal cells, but also in some (1%–2%) endothelial lymphatic cells and myoid cells. This may indicate that both of these cell types are also constitutively equipped to perform steroidogenesis or that they are precursor cells undergoing differentiation. Taken together, changes in the number of Leydig cells in our animal model appeared more likely to be dependent on the transformation of precursor cells than on division of preexisting mature Leydig cells.

Key words: Testis, rat, development, Leydig cells, steroidogenesis, pituitary hormones.

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In the rat testis, the population of "adult" Leydig cells is known to arise, at puberty, from undifferentiated precursors: the so-called precursor mesenchymal cells (Saez, 1994). In an elegant morphological study using the dissector method and covering the period from birth until sexual maturity, Hardy et al (1989) and Benton et al (1995) have shown that from day 14 to day 28, Leydig cells are formed by recruitment of precursor mesenchymal cells, the latter population decreasing in the same proportion as that of adult Leydig cells. From day 28 to day 56, new Leydig cells appear by division of Leydig cells themselves. Furthermore, mesenchymal cells are also known to be precursors of the myoid cells present in the tubular wall (Leeson and Leeson, 1963; Russell et al, 1989).

These morphological modifications of the testicular interstitium, as well as major biochemical and functional changes (in particular the increase of testosterone synthesis by Leydig cells), are controlled by pituitary hormones, the circulating levels of which increase at the onset of puberty (Dussault et al, 1977; Ketelslegers et al, 1978).

From this perspective, we have investigated, in immature hypophysectomized rats, the effects of 7-day treatments by pituitary hormones on the proliferation and differentiation of interstitial cell subpopulations. The animals used in this experiment were treated 2 days after hypophysectomy, at a time when the testis of the hypophysectomized rats is still closed functionally to that of intact animals. Cell proliferation in the testicular in-

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terstitium was measured using *in vivo* bromodeoxyuridine (BUdR) incorporation followed by immunodetection using monoclonal antibodies and immunogold silver staining.

Steroidogenic enzymes (cytochrome P450 cholesterol side-chain cleavage enzyme [$P450_{SCC}$], 3β-hydroxy steroid dehydrogenase [3β-HSD], and 17α-hydroxylase) could be used to distinguish interstitial cells equipped to produce androgens from those that were not. The presence of these three steroidogenic enzymes among the various interstitial cell types was detected using specific antibodies against the enzymes. Special care was also taken to distinguish Leydig cells from macrophages, which they closely resemble under light microscopy (Mendis-Handagama et al, 1990) and which are numerous in the rat testis (Niemi et al, 1986). Accounting for macrophage proliferation is essential because macrophages possess high affinity receptors for pituitary hormones (Yee and Hutson, 1985) and are known to proliferate under different hormonal treatments (Raburn et al, 1991; Dombrowicz et al, 1992; Chen and Johnson, 1993; Gaytan et al, 1994a,b, 1995).

Materials and Methods

Hormones

Human luteinizing hormone (lutropin; hLH) was prepared according to Closset et al (1975) and Closset and Hennen (1989). The latter protocol includes a final immunoaffinity chromatography step using anti-thyroid-stimulating hormone (anti-TSH) and anti-follicle-stimulating hormone (anti-FSH) antibodies immobilized on Affigel 10 (BioRad, Richmond, California). The immunological potency of the preparation was 8,100 IU/mg as measured against International Standard 68/40. Porcine follicle-stimulating hormone (follitropin; pFSH) was purified according to Closset and Hennen (1978, 1989). The last step was an immunoaffinity chromatography using anti-LH antibodies immobilized on Affigel 10. The immunological potency of the preparation is 160 times higher than that of the reference preparation NIH-FSH-P1. Human prolactin (hPRL) was purified according to Hodgkinson and Lowry (1981). Its immunological potency was 39 IU/mg using the International Standard 81/541. Rat growth hormone (rGH) was prepared according to Closset et al (1983) with slight modifications. Its immunological potency was equivalent to that of reference preparation NIADDK rGH 1–5.

Cross-contaminations of each pituitary hormone preparation were measured using specific radioimmunoassays and radio-receptor assays (Vandalem et al, 1975; 1979; Maghuin-Rogister et al, 1978; Closset et al, 1983). These cross-contaminations never exceeded 0.001% by weight for all hormonal preparations tested. The chemical purity of pFSH and hLH was tested by a combination of techniques: dissociation and separation of the α and β subunits by reverse-phase high-performance liquid chromatography (HPLC), followed by sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) of protein fractions according to protocols already described (Closset and Hennen, 1989). On the basis of these analytical methods, LH and FSH preparations appeared to be virtually pure and devoid of contamination with proteins of pituitary origin that were immunologically different from pituitary hormones.

Animals

Wistar rats, hypophysectomized at 19 days of age, were obtained from Iffa Credo (Lyon, France). The rats were housed under standard conditions with delivery of food and water (supplemented with 9 g/L NaCl) *ad libitum*. They were randomly divided into five groups of five animals. Treatments were initiated 2 days postoperatively, at 21 days of age. Five rats of the same age were also taken as controls.

Animals were treated daily for 7 days by s.c. injections of either saline or 10 μg hLH, 32 μg pFSH, 10 μg hPRL, or 50 μg rGH. The chosen concentrations had produced maximum biological effects on the testis of immature rats, as previously shown in dose–response experiments (Closset and Hennen, 1989; Teerds et al, 1989; Closset et al, 1991; Dombrowicz et al, 1992). The animals were killed 24 hours after the last injection. Two hours before sacrifice, they were injected i.v. with 50 mg/kg body weight 5-bromodeoxyuridine (BUdR) and 75 mg/kg fluorodeoxyuridine (FUdR) (Dombrowicz et al, 1988) in order to measure cell proliferation. The animals were anesthetized and testes were fixed *in vivo* by intracardiac perfusion with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, according to Mendis-Handagama et al (1990). Testes were removed and post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 hours at 4°C after incision of the albuginea, then washed overnight in 50 mM NH_4Cl and 2% sucrose, in Na_2HPO_4, pH 7.4 (Beckstead, 1985). The left testis was used for histological analyses.

Animals were weighed before and after treatment and the average body weight gain determined. Animals exhibiting a body weight gain exceeding 0.5 g/day were eliminated from the experimental series.

Histology

Testes were dehydrated in acetone (Van Goor et al, 1988), then embedded in glycolmethacrylate Technovit 7100 (Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Consecutive 2-μm sections were used after either periodic acid–Schiff (PAS) staining, immunodetection of steroidogenic enzymes, or simultaneous detection of acid phosphatase activity (Barka and Anderson, 1962) and cell proliferation. For the latter procedure, acid phosphatase was revealed first; then the sections were extensively washed with distilled water and processed according to Thiry and Dombrowicz (1988) for detection of incorporated BUdR. Briefly, sections were hydrolyzed for 30 minutes at room temperature in 3.5 N HCl, then neutralized in 0.1 M $Na_2B_4O_7$, pH 8.5, for 2 minutes, and finally washed in phosphate-buffered saline (PBS). Anti-BUdR (Becton-Dickinson, Mountain View, California) was detected by immunogold and silver enhancement (Amersham, Buckinghamshire, UK) according to the manufacturers' instructions. For immunodetection of steroidogenic enzymes, the following antibodies were used: rabbit anti-3β-HSD, rabbit anti-$P450_{SCC}$ (gifts from Dr. F. Labrie,

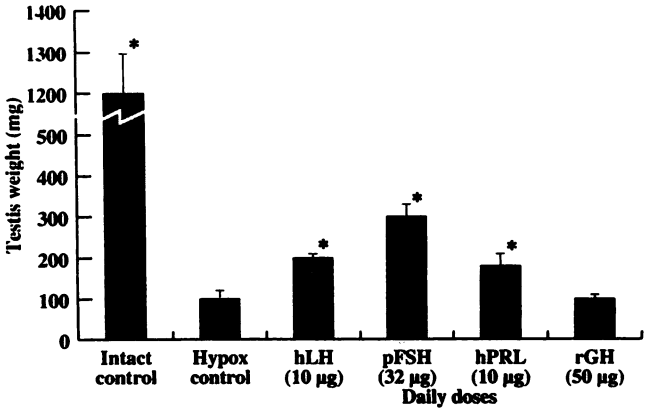

FIG. 1. Testis weight of hypophysectomized rats after 7 days of treatment with different pituitary hormones. The animals were injected daily with 0.15 M NaCl (control), or 10 μg hLH, 32 μg pFSH, 10 μg hPRL, or 50 μg rGH. Animals were killed 24 hours after the last injection. Values are means ± standard error of the mean (SEM) ($n = 5$). An asterisk (*) indicates significantly different from hypophysectomized control ($P < 0.05$).

Laval, Canada and Dr. J. Orly, Jerusalem, Israel, respectively), and rabbit anti-P450 17α-hydroxylase ($P450_{17\alpha}$) (Oxygene, Dallas, Texas). These antibodies have been described elsewhere (Farkash et al, 1986; Luu The et al, 1990). Immunohistochemical detection was performed according to Beckstead (1985), except that the peroxidase-conjugated secondary antibody was replaced by a colloidal gold-coupled antibody (Amersham, Belgium). In controls, consecutive sections were used, and either the substrate of the enzyme or the primary antibody was omitted. All slides were counterstained with Carrazi's hematoxylin.

The different interstitial cell types (Leydig cells, macrophages, precursor mesenchymal cells, myoid cells, endothelial lymphatic cells, capillary endothelial cells, and pericytes) were identified after PAS staining and classified according to Hardy et al (1989). Macrophages were also identified by their acid phosphatase activity. The number of cells of each type was expressed per 1,000 Sertoli cells, as in Heller et al (1971). Germ cells were identified after PAS staining and counted according to Dombrowicz et al (1992). Photographs were taken using an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with a 63× objective (0N 1.40) and Ektar 25 ASA film (Eastman Kodak, Rochester, New York).

Results were statistically analyzed by variance analysis. Simultaneous confidence intervals were determined to investigate possible differences between groups (Scheffé test). Results were considered to be significant at the 5% level.

Results

Hormones

The gonadotropin preparations used in the present study retained high biological activity and were found to have negligible contamination by the known pituitary hormones. Moreover, no contaminant with a molecular weight different from those of the α and β subunits was detected after reverse-phase HPLC, followed by SDS-PAGE, as described by Closset and Hennen (1989). The criteria of purity for LH and FSH are difficult to establish because glycoprotein hormones are known to be naturally heterogeneous both in their peptide backbone and their polysaccharide side chains (Maghuin-Rogister et al, 1975). Moreover, amino-terminal microheterogeneity has been demonstrated for LH and FSH from various species (Shome and Parlow, 1974). Based on these analytical criteria, the LH and FSH preparations used in the present study were free of any detectable contamination.

Testicular Weight

Luteinizing hormone (LH), FSH, and PRL produced 1.8-, 2.3-, and 1.6-fold increases in testicular weight as compared to hypophysectomized control animals. GH treatment did not affect this parameter (Fig. 1).

Histological Aspects

Figure 2 shows glycolmethacrylate sections in which the different cell types of the rat testis were identified according to criteria established by Hardy et al (1989). To accurately identify testicular macrophages from Leydig cells, two different techniques were used: the PAS technique, which stains the macrophage cytoplasm pink, and an enhancement of endogenous acid phosphatase activity, producing a red precipitate on macrophage cytoplasm (Fig. 3g).

In intact animals, the Leydig cell nucleus appeared oval or round, with the characteristic rim of heterochromatin, heterochromatin clumps scattered through the nucleus, and a prominent nucleolus. Seminiferous tubules contained spermatids (Fig. 2a). Hypophysectomized rat testes showed signs of major regression; the cell nucleus was smaller and more irregular in shape. The same was true of cells of the interstitium and of the few cells present in the tubules (Fig. 3b).

Luteinizing hormone treatment caused interstitial hyperplasia; numerous mitotic figures were present (Fig. 3c). Leydig cells with round nuclei were observed in clumps, often near the blood vessels. Precursor mesenchymal cells were numerous. As in the intact rat, the shape of the nucleus appeared variable in these cells (slightly fusiform, elliptical or round). Their chromatin displayed various intermediate stages of condensation, between the rather homogenous aspect observed in endothelial lymphatic cells and the pattern observed in Leydig cells. Cells with an elongated nucleus seemed closer to the tubular wall, whereas cells exhibiting more condensed chromatin were found near the blood vessels. After FSH treatment (Fig. 2d), the few Leydig cells present seemed to be very differentiated. Tubules were voluminous, with prominent Sertoli cells and germ cells up to the late pachytene spermatocyte stage. The effect of PRL on the

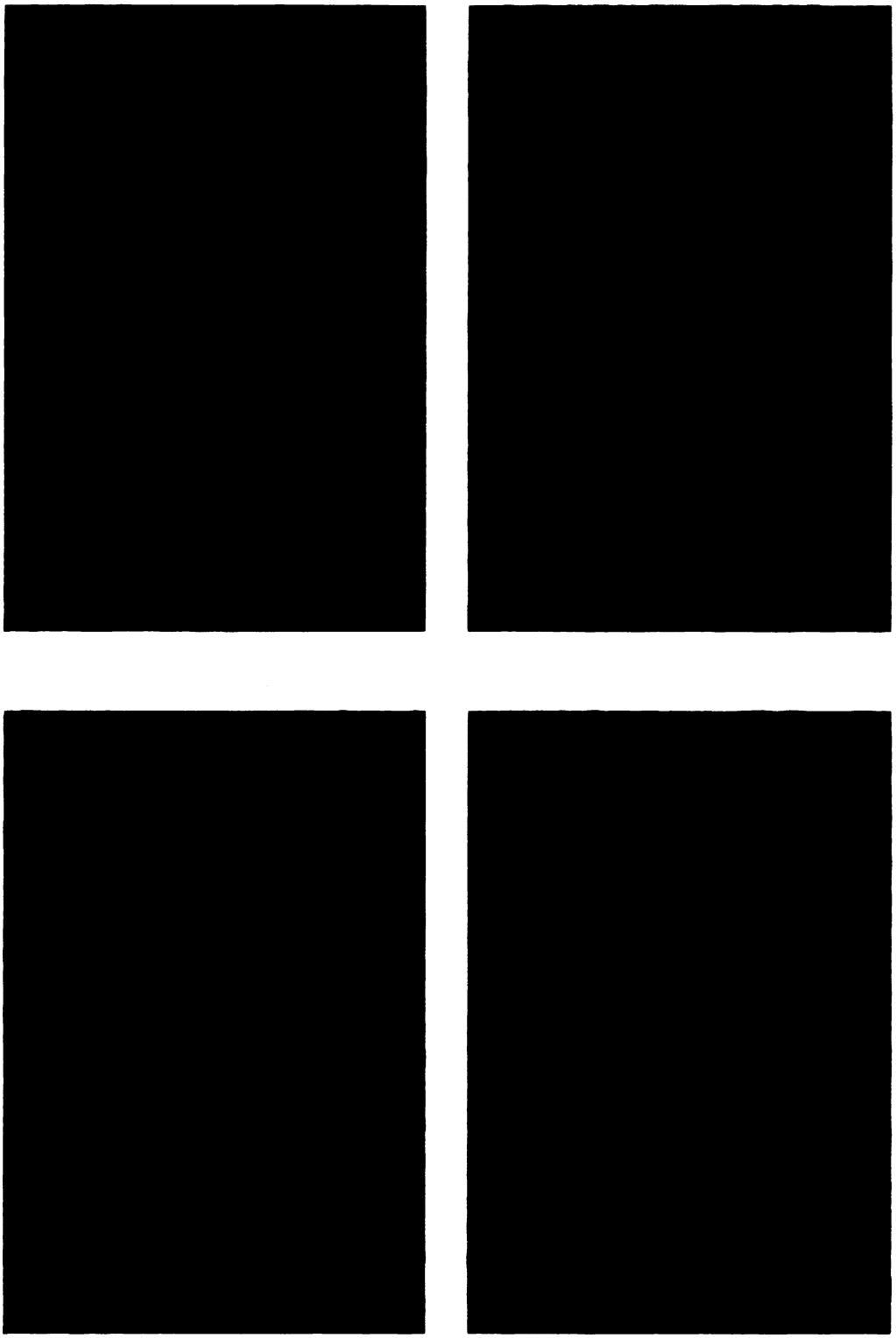

FIG. 2. Testicular histology of normal and hypophysectomized rats. (**a**), Normal rat testis; (**b**), hypophysectomized rat testis; (**c**), testis of hypophysectomized rat treated with hLH (10 μg daily); and (**d**), testis of hypophysectomized rat treated with pFSH (32 μg daily). Magnification, 4,720× (**a** and **c**), 2,360× (**b**), and 9,440× (**d**). Capital letters denote cell types as follows: A, capillary endothelial cell; B, pericyte; C, macrophage; D, Leydig cell; E, precursor mesenchymal cell; F, endothelial lymphatic cell; G, myoid cell; H, Sertoli cell; I, spermatogonia; J, primary spermatocyte (early pachytene); K, primary spermatocyte (late pachytene); and L, spermatid.

cells of the interstitium resembled that of LH but was less pronounced.

Interstitial Cell Subpopulations

The number of cells in each subpopulation was expressed per 1,000 Sertoli cells, as in Heller et al (1971), because the number of Sertoli cells no longer increases after the 15th day of life (Wang et al, 1989) and, after short-term hypophysectomy, the Sertoli cell number was not significantly affected (Ghosh et al, 1992).

The assay of the acid phosphatase activity showed that when compared to intact 30-day-old rats, the hypophysectomized rats of the same age displayed a twofold decrease in the number of macrophages (Fig. 4). Treatment with rat GH did not modify this number. On the other hand, treatment with LH, FSH, and PRL increased the number of acid phosphatase-labeled macrophages by about 1.5-, 1.4-, and 1.3-fold, respectively, compared to hypophysectomized control animals (Fig. 4).

The number of morphologically recognizable Leydig cells was 10 times lower in hypophysectomized animals, compared to controls. There were also fewer precursor mesenchymal cells (Fig. 5). After treatment with LH, PRL, or GH, the number of Leydig cells was 8, 3, or 1.8 times, respectively, as high as that in hypophysectomized control testes. There were 1.9 and 1.5 times as many precursor mesenchymal cells after treatment with LH or PRL, respectively, as in hypophysectomized controls. All hormonal treatments, especially with PRL and GH, decreased the number of endothelial lymphatic cells 1.5-fold when compared to hypophysectomized control animals. LH treatment increased the number of myoid cells, whereas all other hormones slightly decreased it (Fig. 5).

Proliferation of Interstitial Cells

Using the double labeling procedure (acid phosphatase activity and BUdR immunodetection), the BUdR incorporation into the testicular macrophages was detected in both intact animals and hypophysectomized rats treated with PRL, LH, or FSH, as reflected by the silver grains over the nuclei of cells with a red-colored cytoplasm (Fig. 3g). The uptake of BUdR was higher after PRL treatment than after LH and FSH supplementation, but no incorporation was detected in hypophysectomized control rats and GH-treated animals. When we assessed the proliferation of other interstitial cell types, no incorporation was detected in the interstitium in hypophysectomized rats. Precursor mesenchymal cells were the predominant cell type labeled (Fig. 3h) after all hormonal treatments. Leydig cells were labeled in intact rats and in hypophysectomized animals treated with LH and PRL. In all but the GH-treated animals, labeled endothelial lymphatic cells were also detected. Myoid cells were labeled in all hormone-treated animals. LH was found mainly to stimulate the proliferation of precursor mesenchymal cells.

Immunoreactivity Towards Enzymes That Stimulate Steroidogenesis

We used specific antibodies to detect the enzymes $P450_{SCC}$, $P450_{17\alpha}$, and 3β-HSD in the various interstitial cell subpopulations. The trypsinization step and the subsequent alteration of chromatin morphology prevented us, however, from being able to distinguish Leydig cells and precursor mesenchymal cells. These two cell types were therefore counted together as a single subpopulation.

Labeling by anti-3β-HSD was detected in all the experimental groups, but its intensity was lower in hypophysectomized controls and GH-treated animals. In the labeled cytoplasm, label-free zones could be clearly distinguished (Fig. 3a). These may have been lipid inclusions, often observed in the Leydig cells of immature animals, or zones that contained no smooth endoplasmic reticulum. There were nearly three times fewer immunoreactive cells in hypophysectomized control animals than in intact rats. In addition to Leydig and precursor mesenchymal cells, a few endothelial lymphatic cells were labeled. LH treatment increased the number of labeled cells 3.5-fold, to a count slightly higher than that in intact rats (Fig. 3b). The number of labeled endothelial lymphatic cells was 38-fold higher than in hypophysectomized control animals. A few myoid cells were also labeled (Fig. 3d). Their cytoplasms appeared black whereas those of control sections were slightly colored (Fig. 3f). PRL treatment produced similar but weaker effects. By contrast, FSH and GH barely affected the number of immunopositive Leydig and precursor mesenchymal cells. FSH did, however, cause the number of labeled endothelial lymphatic cells to increase 11-fold over the hypophysectomized control. The effect of GH was similar but much weaker (Fig. 7a).

No $P450_{17\alpha}$ or $P450_{SCC}$ immunoreactivity was detected in hypophysectomized, FSH-treated, or GH-treated animals. In intact, LH-treated, and PRL-treated rats, labeling of $P450_{SCC}$ and $P450_{17\alpha}$ was weaker than labeling of 3β-HSD, but in all three cases, the number of immunopositive cells followed the same pattern of variation with hormonal treatment (Fig. 3c). In normal and LH-treated rats, moreover, fewer Leydig and precursor mesenchymal cells were labeled by the anti-$P450_{SCC}$ and $P450_{17\alpha}$ antibodies than by the anti 3β-HSD antibody. On the other hand, more myoid cells tested immunopositive for $P450_{SCC}$ than for 3β-HSD in LH- and PRL-treated rats. In intact animals, 1%–2% of myoid cells were also labeled with both anti-$P450_{SCC}$ antibodies (Fig. 7b). Because the labeling of $P450_{17\alpha}$ was much weaker than the labeling of $P450_{SCC}$, only the total numbers of interstitial cells immunopositive for this enzyme were counted (Fig. 7c).

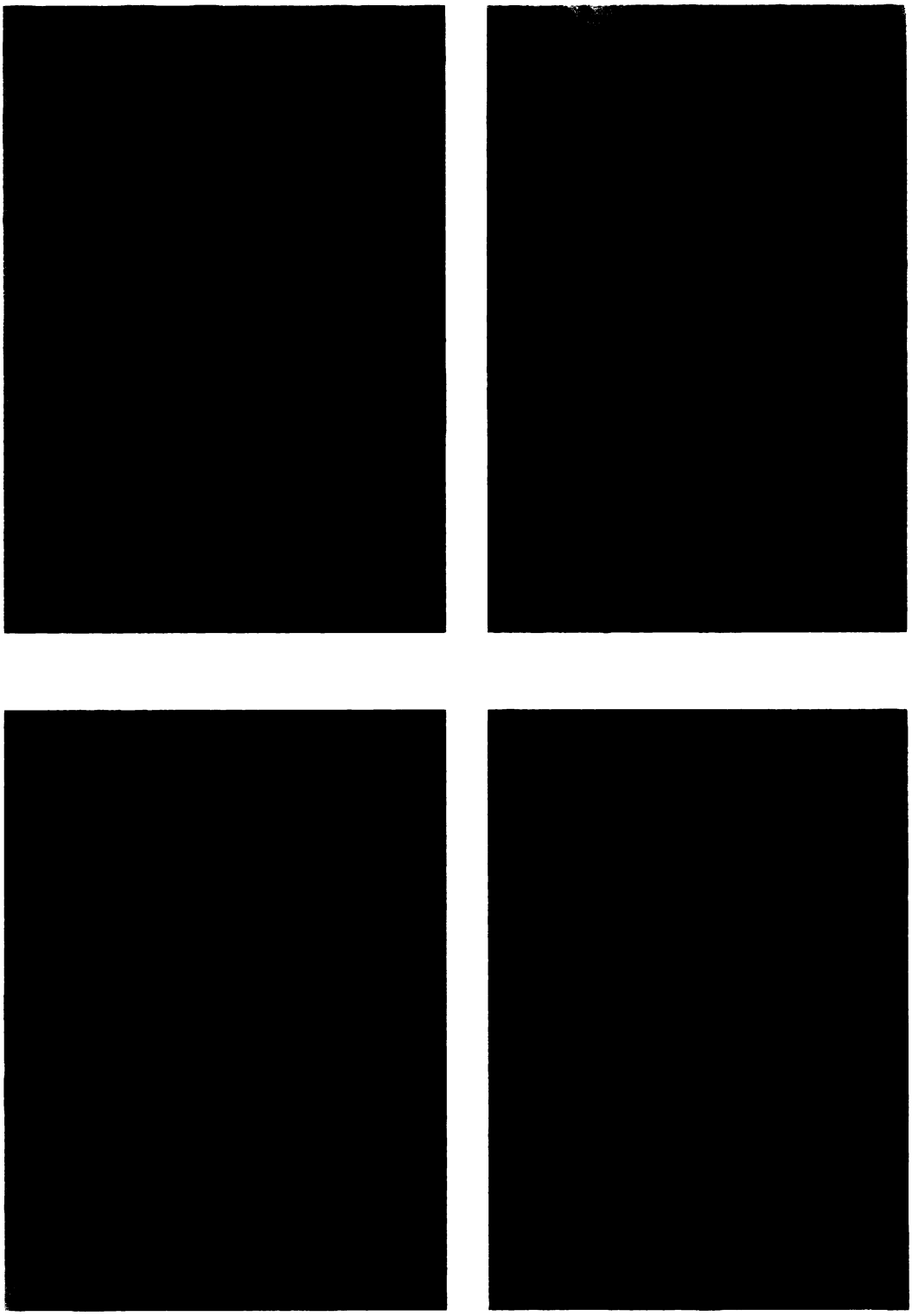

FIG. 3. Interstital cell proliferation and their immunoreactivity for steroidogenic enzymes. (**a**), Immunodetection of 3β-HSD in the interstitium in an intact rat. Leydig and precursor mesenchymal cell cytoplasm is strongly positive. Cytoplasmic inclusions devoid of silver deposits are very visible (magnification, 4,729×). (**b**), Immunodetection of 3β-HSD in the interstitium in a hypophysectomized rat treated with 10 μg hLH for 7 days. Numerous Leydig and precursor mesenchymal cells are labeled. (**c**), Immunodetection of $P450_{scc}$ in the interstitium in an intact rat. Leydig and precursor mesenchymal cells are faintly labeled. (**d**), Immunodetection of 3β-HSD in a hypophysectomized PRL-treated rat. Labeling is clearly visible in the cytoplasm of myoid cells. The scale bars represent 10 μm.

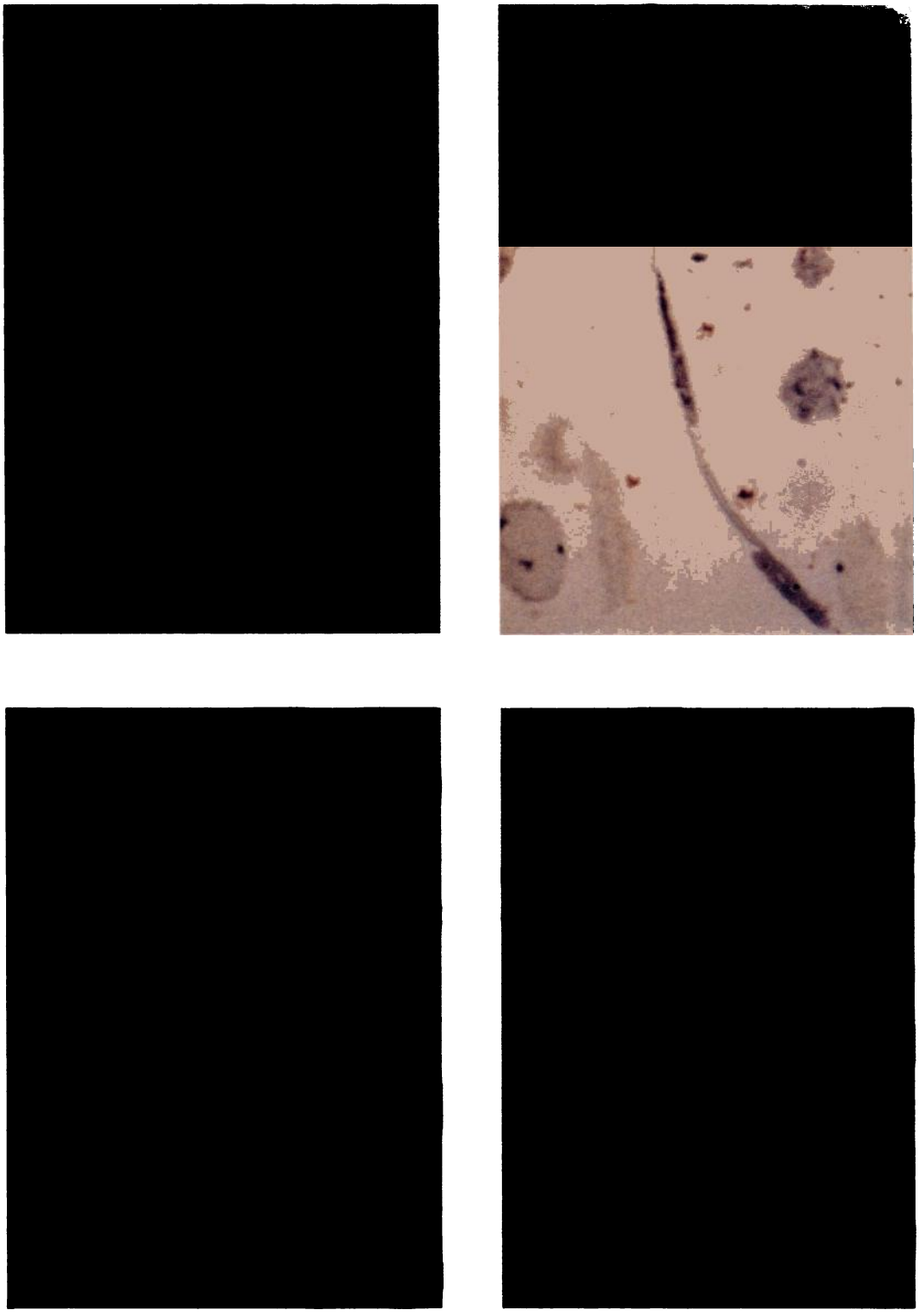

FIG. 3. Continued. (**e**), Negative control of the immunodetection of $P450_{scc}$ in the interstitium in an intact rat. (**f**), Negative control of the immunodetection of 3β-HSD in a hypophysectomized PRL-treated rat. No labeling was detected in the cytoplasm of myoid cells. (**g**), Acid phosphatase labeling and BUdR immunodetection in macrophages (▲) in a 30-day-old intact rat. (**h**), Acid phosphatase labeling and BUdR immunodetection in macrophages (▲) and precursor mesenchymal cells (△) in a hypophysectomized PRL-treated rat. The asterisk (*) indicates a macrophage mitotic figure.

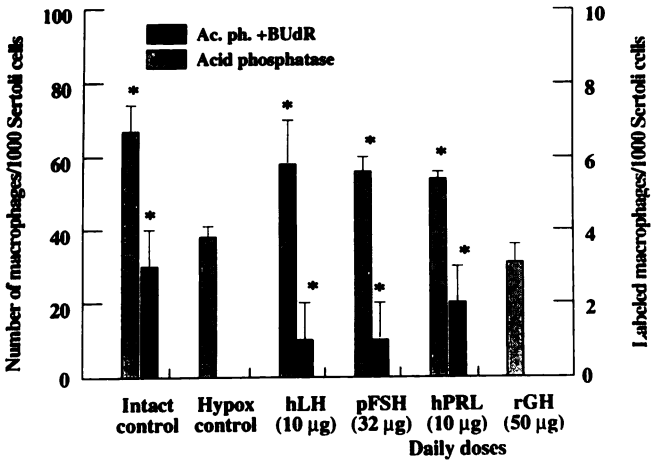

FIG. 4. Effect of different pituitary hormones on macrophage number and on BUdR incorporation in macrophages. Cell numbers are expressed per 1,000 Sertoli cells. Values are means ± SEM (n = 5). Asterisk (*) indicates significantly different from hypophysectomized control ($P < 0.05$).

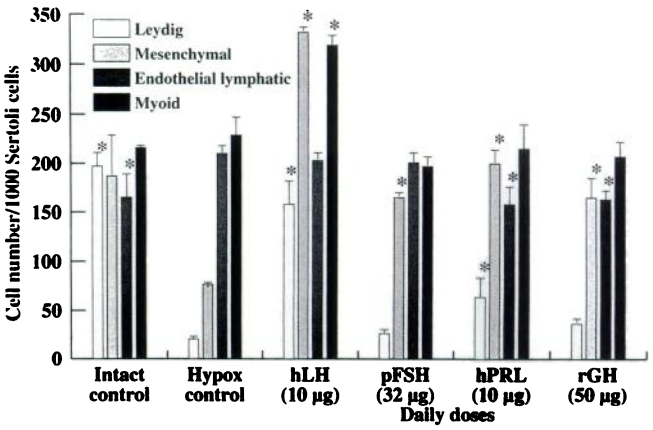

FIG. 5. Effect of different pituitary hormones on cell numbers in different interstitial cell subpopulations. Cell numbers are expressed per 1,000 Sertoli cells. Values are means ± SEM (n = 5). Asterisk (*) indicates significantly different from hypophysectomized control ($P < 0.05$).

Discussion

Methodological Considerations

We investigated the effects of a 7-day supplementation highly purified individual pituitary hormones on the various cell types present in the testicular interstitium of immature rat 2 days after hypophysectomy. This experimental model, although imperfect, permitted us to investigate the capacity of each single hormone to block the degenerative processes induced by hypophysectomy. This model does not reproduce the effects of a single hormone defect (as would gene targeting techniques or mutant strains), nor does it reveal the synergistic effects produced by two or more hormones (multiple supplementations would be required to allow such data to be collected). In particular, we did not study the synergistic effects of any of these hormones with TSH, which, to some extent, regulates testicular function and which is missing in our model (Palmero et al, 1989; Hardy et al, 1993; Janini et al, 1994).

For the sake of clarity, we will use the term "proliferation" when the cell number or the BUdR incorporation in any given population is increasing in an experimental group, compared to corresponding data in the untreated hypophysectomized animals. Likewise, an increase in the ratio between adult-type Leydig cells and their putative precursors after any hormonal treatment will be termed "differentiation."

Throughout this paper, the abundance of the various cell populations was expressed as a number of cells per 1,000 Sertoli cells. This means of reporting data is valid because the number of Sertoli cells does not vary after day 10 postnatally and does not decrease for the first 6 days after hypophysectomy (Ghosh et al, 1992).

Cell Number and Proliferation

Macrophages—Testicular macrophages were identified either by PAS staining or an assay of endogenous acid phosphatase activity. In our hands, the latter appeared to be more sensitive. LH, FSH, and PRL were able to increase cell number and BUdR incorporation in interstitial macrophages. The effects of LH on those cells have already been documented in newborn (Raburn et al, 1991) and prepubertal and adult animals (Gaytan et al, 1994 a,b, 1995). The effects of LH could be indirect and due either to an increased secretion of cytokines and growth factors by adjacent Leydig cells or to an induced increase in vascular permeability and reduced blood flow, thus allowing greater interstitial concentrations of circulating growth factors (Van Vliet et al, 1988; Hedger et al, 1990). Direct action of LH would require the existence of thus-far unreported macrophagic LH receptors or, more simply, Ley-

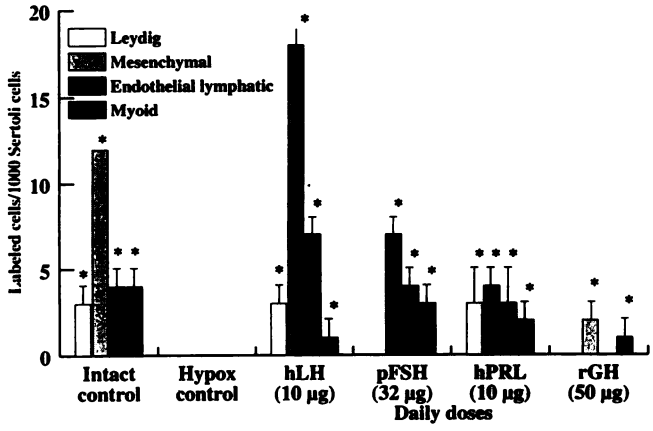

FIG. 6. Effect of different pituitary hormones on BUdR incorporation in interstitial cells. Cell numbers are expressed per 1,000 Sertoli cells. Values are means ± SEM (n = 5). Asterisk (*) indicates significantly different from hypophysectomized control ($P < 0.05$).

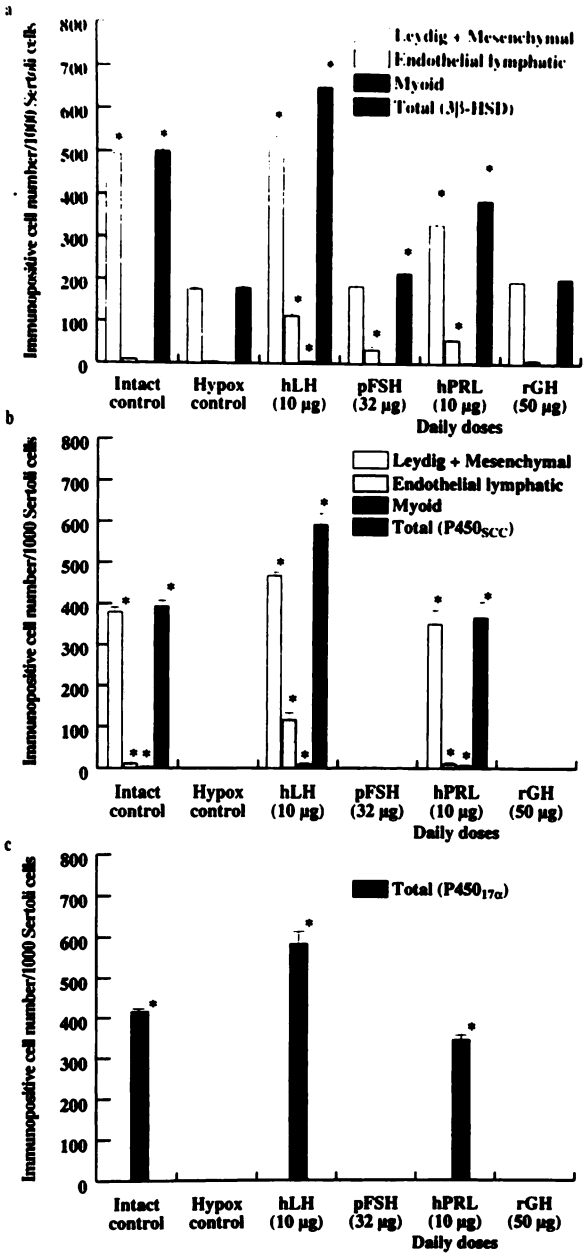

FIG. 7. Effect of different pituitary hormones on cell numbers of interstitial cells immunoreactive for 3β-HSD **(panel a)**, $P450_{scc}$ **(panel b)**, and $P450_{17\alpha}$ **(panel c)**. Cell numbers are expression per 1,000 Sertoli cells. Values are means ± SEM (n = 5). Asterisk (*) indicates significantly different from hypophysectomized control ($P < 0.05$).

dig cell factors, because communication exists between macrophages and Leydig cells (Saez, 1994). By contrast, Yee and Hutson (1985) have demonstrated the presence of FSH receptors on macrophages. Furthermore, FSH has been shown to increase lactate metabolism in macrophages isolated from testis of breeding hamster (Mayerhofer et al, 1992). PRL is known to stimulate peritoneal and testicular macrophages (Chen and Johnson, 1993; Gaytan et al, 1994b) via specific receptors (Gala and Shevach, 1993). In contrast to a previous report on adult hypophysectomized rat testis (Gaytan et al, 1994b), we were unable to demonstrate any effect of rGH on the macrophage population in immature animals.

Leydig Cells and Their Putative Precursors—Luteinizing hormone increases the number and the BUdR incorporation of all interstitial cell populations except the endothelial lymphatic cells. In particular, Leydig cells treated with LH are almost as numerous as in the intact testis. Because precursor mesenchymal cells appear to be highly proliferative and display a very heterogeneous morphology (in the shape of the cytoplasm and nucleus, and in the chromatin condensation pattern), intermediate between Leydig and endothelial lymphatic cells, one could reasonably argue that they would be progenitors not only of the former but also of the latter cell type. Such an intermediate phenotype was reported by Kerr and Sharpe (1985a) after FSH treatment of hypophysectomized rats. LH acts directly via specific receptors not only on Leydig cells but also on precursor mesenchymal cells. The effects of LH on precursor cells might also be indirect and mediated through testosterone produced by Leydig cells, because precursor mesenchymal cells possess androgen receptors. Indeed, LH and androgens have been shown to promote their differentiation *in vitro* as well as *in vivo* (Hardy et al, 1990; Leon et al, 1990; Shan et al, 1995). The effects of LH on myoid cells, although slight, are likely to be mediated through androgen synthesis by Leydig cells (Anthony et al, 1989). As previously reported (Leeson and Leeson, 1963; Russell et al, 1989), it seems likely that myoid cells also arise from precursor mesenchymal cells.

Prolactin treatment stimulates BUdR incorporation in all cell populations and increases Leydig and precursor mesenchymal cell number but decreases peritubular cell number. This could be due to the transformation of peritubular cells into precursor mesenchymal cells. As stated for LH, the effects of PRL on Leydig and probably on precursor mesenchymal cells are direct, and they are androgen mediated in the case of myoid cells (Charreau et al, 1977).

Follicle-stimulating hormone barely affects Leydig cell number and only slightly increases cell numbers in other interstitial cell populations. Although there is no incorporation of BUdR into Leydig cells and there is only half as much BUdR incorporation following FSH treatment as there is following LH treatment in precursor mesenchymal and peritubular cells, FSH is the most potent stimulus for BUdR incorporation into myoid cells. Because the interstitium is lacking in specific FSH receptors, this hormone acts through paracrine secretion of Sertolian factors whose existence is now well established (Skinner, 1991).

Growth hormone increases precursor mesenchymal (two times) cell proliferation, but exerts no or opposite

effects on the other interstitial cell populations. Likewise, its effects on BUdR incorporation are only minor. Leydig cells have been shown to possess GH receptors (Mathews et al, 1989). In addition GH-induced insulin-like growth factor I (IGF-1) synthesis by Leydig cells or hepatocytes (Closset et al, 1989; Lin et al, 1990, 1993) stimulates those processes by interaction with the cognate receptors present on interstitial cells (Handelsman et al, 1985).

Immunodetection of Steroidogenic Enzymes: Effects of Pituitary Hormones—In FSH-treated, GH-treated, and hypophysectomized control rats, we detected no reaction with specific anti-$P450_{SCC}$ or anti-$P450_{17\alpha}$ antibodies. In the interstitial cells of such animals, the amounts of these enzymes are probably below the detection threshold of our immunohistochemical methods. Although the corresponding enzyme activities have been detected in interstitial cells, even in those of hypophysectomized animals, such detection required the use of tritiated substrates (Skinner, 1991). In the other experimental groups, immunoreactive cells were found, and the number of cells testing positive for $P450_{SCC}$ and $P450_{17\alpha}$ was roughly the same.

In intact and LH-treated animals, the higher number of Leydig and precursor mesenchymal cells testing immunopositive for steroidogenic enzymes as compared to morphologically recognizable cells could be due to an erroneous identification of these cells after trypsinization. Our results show that not only Leydig cells but also precursor mesenchymal, endothelial lymphatic, and myoid cells contain steroidogenic enzymes. Myoid cells have never before been implicated in steroidogenesis, although 3β-HSD activity has been detected in endothelial lymphatic cells (Wiebe, 1976), and human chorlanic gonadotropin (hCG) has been shown to induce testosterone production in prepubertal boys (Chemes, 1986) and in precursor mesenchymal cells isolated from boys with complete androgen insensivity syndrome (Chemes et al, 1992). However, more direct experiments, such as the detection of enzymatic activity on frozen tissue sections, are needed to confirm the presence of steroidogenic enzymes in endothelial lymphatic and myoid cells.

We have also shown that LH, among the pituitary hormones tested, is the most effective in increasing the number of cells testing immunopositive for the three steroidogenic enzymes in all cell populations of the interstitium. This result strengthens the main role played by LH, not only on interstitial cell proliferation but also on the production of steroidogenic enzymes by the rat testis (Payne, 1990). PRL exerts similar but lesser effects on the same cell types. FSH (and GH, to a lesser extent) mainly affects the number of endothelial lymphatic cells testing positive for 3β-HSD. As suggested by Kerr and Sharpe (1985a,b), the action of FSH in hypophysectomized animals could be restricted to a population of fusiform cells localized near tubules.

In conclusion, taken together, our results suggest that in the immature hypophysectomized rat the number of Leydig cells present in the testis is more likely dependent on the transformation of precursor cells than on division of preexisting mature Leydig cells. Indeed, we have shown that cells not morphologically recognized as Leydig cells possess the enzymes that perform steroidogenesis, and we have shown that the different cell types of the interstitium proliferate in response to specific hormonal stimulation. By comparing the proliferative response of each cell type to pituitary hormone stimulation with the effects of pituitary hormones on the total number of cells in each subpopulation of the interstitium, we have shown that these hormones promote differentiation of some cells and in some cases cause reversal of differentiation.

Our results shed some light on the roles of the various pituitary hormones in interstitial maturation during pubertal development. However, because the interstitium of immature rats regresses spontaneously, even after short-term hypophysectomy (Ghosh et al, 1992), a better understanding of the pituitary control of testicular maturation at puberty might be gained from experiments based on homogeneous subpopulations of interstitial cells in culture.

Because hormone and growth factor receptors are involved in all cellular differentiation mechanisms, anti-receptor antibodies would be an ideal tool for probing the hormonal control of testicular maturation. These antibodies might also be useful to determine whether pituitary hormone receptors are expressed on differentiated interstitial cells other than Leydig cells.

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