

## Alterations of Testicular Function After Induced Autoimmune Orchitis in Rats

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**ABSTRACT:** The endocrinological profile of animals with experimental autoimmune orchitis (EAO) has not been sufficiently explored. With this purpose orchitis was induced in adult rats by active immunization with testicular homogenate (TH) and adjuvants. Animals were sacrificed 50 or 80 days after the first immunization. Forty-three percent of rats immunized with TH developed orchitis. Different degrees of cell sloughing and atrophy of the seminiferous tubules and numerous macrophages and lymphocytes in close association with Leydig cells were seen. A significant increase in the number of Leydig cells was observed in rats with orchitis killed at 50 and 80 days. An enhanced number of interstitial non-Leydig cells was also detected in rats with testicular damage killed at 80 days. Levels of serum follicle-stimulating hormone (FSH) were two- to threefold high-

er in rats with EAO compared to concentrations detected in other groups. Moreover, rats with orchitis had significantly increased testicular testosterone. Serum luteinizing hormone (LH) did not change in animals of any group. *In vitro* studies showed an increase in the basal and human chorionic gonadotropin (hCG)-stimulated testosterone production in rats with EAO. The increase in testicular steroidogenesis without a concomitant enhancement in serum LH levels detected in rats with autoimmune orchitis suggests the existence of local control mechanisms.

Key words: Orchitis, steroidogenesis, Leydig cells, testosterone, testicular macrophages, autoimmunity.

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Despite numerous reports on histopathologic and immunologic aspects of experimental autoimmune orchitis (EAO), the endocrinological profile of animals with this disease has not been sufficiently explored. We previously demonstrated (Doncel et al, 1989) a temporal relationship between histopathological changes in the testis, and a cellular and humoral immune response during the development of autoimmune orchitis in rats. More recently, we have also described (Doncel and Lustig, 1991; Lustig et al, 1993) variations in the lymphocyte subsets and Ia<sup>+</sup> cells in the lymph nodes, as well as in the testis intertubular cell infiltrates of rats with EAO, that are probably involved in the pathogenesis of testis damage.

It has long been recognized that the differentiation and function of Leydig cells is regulated by luteinizing hormone (LH). However, numerous reports (Sharpe, 1984; Saez et al, 1987; Verhoeven, 1992) highlight the wide variety of cell-cell interactions between interstitial and tubular compartments involved in local control mechanisms of the Leydig function.

The induction of seminiferous tubule damage in adult rats in different experimental models, such as cryptorchidism, irradiation, and vitamin A deficiency, results in changes in Leydig cell morphology and function. It has been suggested that Sertoli cell-secreted factors are probably responsible for such changes. It has also been reported that different products of interstitial lymphocytes and macrophages can influence Leydig cell function (Skinner, 1991).

The aim of the present study was to assess *in vivo* and *in vitro* testicular steroidogenesis of rats undergoing autoimmune orchitis induced by active immunization with a testicular homogenate, and to correlate these data with the testicular damage.

### Materials and Methods

#### Animals

Male Sprague-Dawley rats 50-56 days old (mean weight 270 g) were used. Animals were housed at 22°C with a 14-hour light: 10-hour dark ratio and were fed standard pellets and water *ad libitum*.

#### Immunization Schedule

Thirty rats (experimental group) were immunized with a testicular homogenate (TH) prepared as previously described (Doncel et al, 1989). Briefly, rat testes were decapsulated, diluted in an

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equal volume of saline, and disrupted in an Omni mixer for 30 seconds. A final concentration of 500 mg/ml wet weight (ww) was obtained. Animals were injected three times with 200 mg (ww) of TH per dose per rat at intervals of 14 days. Antigen (0.4 ml) emulsified with 0.4 ml of complete Freund's adjuvant (CFA) was injected intradermally in footpads and at multiple sites near ganglionic regions. The first two immunizations were immediately followed by an i.v. injection of 0.5 ml of *Bordetella pertussis* (Bp) (strain 10536; Instituto Malbrán, Buenos Aires, Argentina) containing  $10^{10}$  microorganisms. After the third immunization,  $5 \times 10^9$  microorganisms were injected i.p. As a control group (C), 12 rats were injected with an emulsion of saline, CFA, and Bp with the same conditions as for the experimental group. Twelve nonimmunized normal rats (N) of the same age as the animals described above were also studied. Rats were killed by decapitation, half of them 50 days and the other half 80 days after the first injection. These intervals were chosen because, as we described previously for Wistar rats (Doncel et al, 1989), they correspond to the onset and maximum severity of testicular lesions, respectively. Blood was collected and sera were stored at  $-20^\circ\text{C}$  for measurement of serum LH, follicle-stimulating hormone (FSH), and testosterone (T). Body and testis weights were determined for each rat. One testis was processed for light microscopy and morphometric analysis, and the other was processed for T content and *in vitro* steroidogenic studies.

#### Morphologic and Morphometric Techniques

For light microscopy, testes were fixed in Bouin's solution, embedded in paraffin, sectioned at three different levels, and stained with hematoxylin-eosin (HE). In order to determine Leydig volume and numerical density, a morphometric study was performed in four animals per group; in this case testes were fixed by perfusion through the testicular artery. The study was performed using paraffin sections (equal section widths) following the method described by Mori and Christensen (1980), with minor modifications. Briefly, sections were analyzed with a  $40\times$  objective, and the ocular ( $12.5\times$ ) was fitted with a quadratic grid having 121 intersections ( $11 \times 11$ ), utilized as counting points. The number of "hits" divided by the total number of points yields the volume density (volume of individual Leydig cells per unit volume testis). The average volume of individual Leydig cells was calculated by dividing the volume density by the numerical density. Counts for numerical density (number of Leydig cells per unit volume) were made at the same time by counting all Leydig cells ( $N_a$ ) occurring within the total grid boundary (an area of  $361,000 \mu\text{m}^2$  on the specimen). Leydig cells were distinguished from other cells of the interstitial tissue by their prominent nuclei with distinct chromatin and conspicuous nucleolus. The total number of grid fields counted for each section was 40, and four animals per group were studied. Average nuclear diameter (D) was measured with a  $100\times$  Zeiss objective and an ocular micrometer calibrated with a stage micrometer (Christensen and Peacock, 1980). The final numerical density or number of Leydig cells per unit volume testis ( $N_v$ ) was then calculated by the Floderus equation (Floderus, 1944):  $N_v = N_a/D + T - 2h$ , where T is the section thickness, h a correction factor to calculate nuclei diameter, and D the average nuclear diameter calculated as described in detail by Mori and Christensen (1980).

The total number of Leydig cells per testis was calculated by multiplying the final numerical density by the testis volume. The latter was calculated as the ratio of testis weight and the specific gravity ( $1.040 \pm 0.004$  standard error of the mean [SEM]), according to Mori and Christensen (1980). Preliminary results obtained in rats with EAO showed us that no major variations in the testicular specific gravity were observed among control and experimental groups. The same procedure was used to obtain the numerical density of interstitial non-Leydig cells (mainly macrophages and lymphocytes). In this case, all interstitial cells, except cells from vessel walls, red blood cells, and Leydig cells, were counted.

#### Hormone Measurements

Serum LH and FSH were measured by double-antibody radioimmunoassay (RIA) as previously described (Baraño et al, 1982). Results are expressed as ng/ml on the basis of the standards rLH RP-2 and rFSH RP-2, supplied by the National Institute for Diabetes and Diseases of the Kidney (NIDDK) Rat Pituitary Distribution Program. The within-assay and interassay coefficients of variation are less than 8% and 13%, respectively, for both assays. The minimum detectable concentrations in the rLH and rFSH assays are 0.2 ng/ml and 1.0 ng/ml of serum, respectively. Serum T was measured by RIA using T ( $1,2\text{-}^3\text{H}[\text{N}]$ ; 60.00 Ci/mmol) from New England Nuclear (Boston, Massachusetts) and specific antibody from Immunotech Diagnostic (Montreal, Canada), as previously described and validated (Suescun et al, 1985). The minimum detectable concentration in the T assay is 12.5 pg/ml.

Testicular T content was also measured by RIA in samples processed as previously described and validated (Suescun et al, 1981). Briefly, pieces of testicular tissue were homogenized in acetone and centrifuged, and supernatants were evaporated to dryness. After resuspension in water, samples underwent a sequence of solvent partitions: water-diethyl ether (1:10, v/v), 70% methanol-hexane (1:1, v/v), and methanol-dichloromethane (1:3, v/v). Samples were evaporated, and the residues were resuspended in buffer and assayed for T by RIA as described above. The within-assay and interassay coefficients of variation were less than 12%. Recovery of the initial tritiated standard was  $70 \pm 4.7\%$  (mean  $\pm$  standard error [SE]). Results were expressed in terms of ng per testis.

#### Assessment of T Production

*In vitro* T production was determined by incubating two hemitestis from each animal, separately, in 2 ml of M-199 medium with 0.1% (w/v) bovine serum albumin and 0.1 mM 1-methylisobutylxanthine (MIX). One sample was incubated in medium alone (basal), and human chorionic gonadotropin (hCG, 100 mIU/ml; Ayerst Laboratory, Montreal, Canada) was added to the other one. Incubations were performed for 4 hours at  $34^\circ\text{C}$  in a 95%  $\text{O}_2$ , 5%  $\text{CO}_2$  atmosphere in a Dubnoff shaking incubator (88 cycles/minute). After incubation, samples were decanted, media centrifuged, and the supernatants stored at  $-20^\circ\text{C}$  until they were assayed for T content by RIA without previous extraction.

### Statistical Analysis

Statistical evaluations were performed by analysis of variance and Tuckey's test for hormonal results, and by the non-parametric Mann-Whitney rank test for morphometric studies.

## Results

### Histopathology and Morphometry

Results of light microscopy showed that 43% of rats immunized with TH developed orchitis. Therefore, within the experimental group (E), subgroups of rats with or without testis lesions (EL and ENL, respectively), were observed. Variations in the body weight of rats from different groups were not detected. No significant differences were observed between the mean values of testis weight of rats from control and ENL groups (N:  $1.96 \pm 0.1$  g, C:  $1.91 \pm 0.1$  g, and ENL:  $1.91 \pm 0.1$  g). However, rats with testis lesions killed at 50 days (EL:  $1.51 \pm 0.4$  g) and 80 days (EL:  $0.9 \pm 0.18$  g) revealed a significant reduction in testis weight in comparison to controls ( $P < 0.01$  and  $P < 0.001$ , respectively). The histopathologic feature of testis damage was almost identical to our previous description for Wistar rats (Doncel et al, 1989), except that a milder interstitial cell infiltrate was observed in the Sprague-Dawley rats. The severity of EAO ranged from a moderate lesion, characterized by the presence of foci of damaged seminiferous tubules, to severe orchitis with large damaged areas. Different degrees of cell sloughing and atrophy were observed, and in severely damaged testes, only spermatogonia and Sertoli cells remained attached to the tubular wall. Vacuolization of Sertoli cell cytoplasm and multinucleated giant cells was frequently seen. Interstitial mononuclear cell infiltrates were usually mild at 50 days, but they showed increased cell density at 80 days. Numerous macrophages and lymphocytes were seen in close association with Leydig cells that exhibited normal morphology. The incidence of animals with severe

orchitis was higher in rats killed after 80 days than in those sacrificed after 50 days (50% and 33%, respectively). None of the rats from normal and control groups showed pathological alterations of the testis (Fig. 1).

Table 1 shows the mean value of Leydig cell volume density and the number of Leydig cells and interstitial non-Leydig cells per testis. Despite the reduction of testis volume observed in rats with testicular damage, the absolute number of Leydig cells per testis was increased in rats with orchitis (EL). This enhancement in cell number was significant for rats killed at 80 days. A similar phenomenon was observed for the interstitial non-Leydig cells per testis in rats with orchitis (EL). No significant differences in the average volume of individual Leydig cells among the different groups of rats were detected (mean value:  $1,253 \pm 157 \mu\text{m}^3$ ).

### Hormone Measurements

As shown in Figures 2 and 3, no significant differences were observed in the FSH serum levels of the N, C, and ENL groups of rats. However, in rats with orchitis (EL) a two- and threefold increase in serum FSH was detected at 50 and 80 days, respectively, compared to data from the previous groups.

Serum concentrations of LH from rats of the experimental groups (EL:  $1.02 \pm 0.08$ , ENL:  $1.17 \pm 0.09$  ng/ml) did not differ significantly from those of the control group ( $1.10 \pm 0.09$  ng/ml) and nonimmunized rats ( $1.32 \pm 0.26$  ng/ml). These data are mean values obtained from rats killed at 50 and 80 days because no differences were detected in these two periods. At 50 days, T serum levels were significantly reduced in rats with orchitis (EL:  $1.30 \pm 0.14$  ng/ml) compared to the values for the other groups (C:  $2.42 \pm 0.27$ , N:  $1.86 \pm 0.11$ , ENL:  $2.18 \pm 0.09$  ng/ml) ( $P < 0.05$ ). However, this reduction was not observed at 80 days (N:  $2.1 \pm 0.10$ , C:  $2.2 \pm 0.28$ , EL:  $2.0 \pm 0.35$ , ENL:  $3.0 \pm 0.46$  ng/ml).

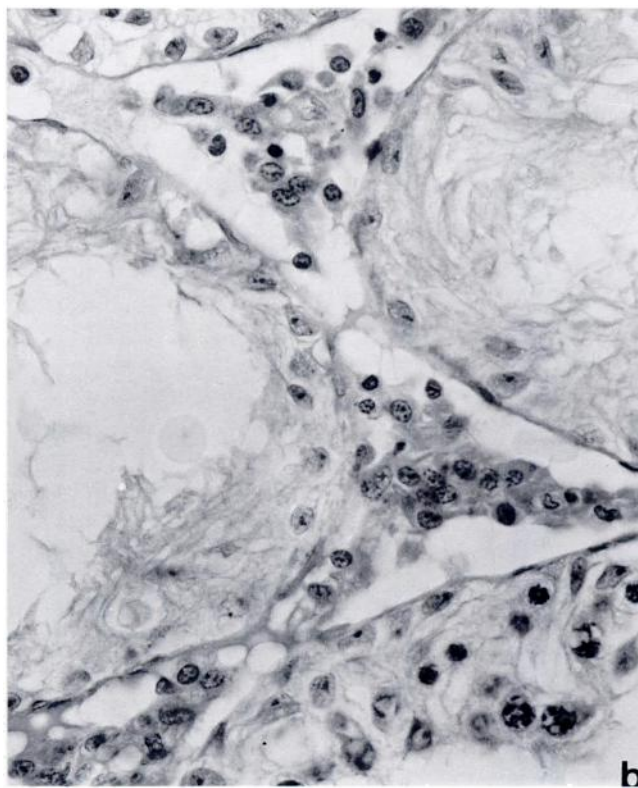
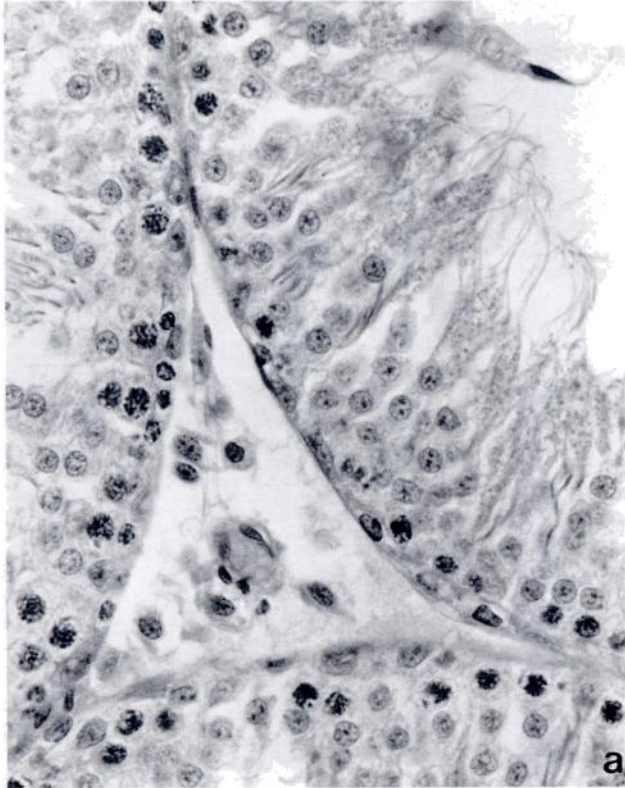
As shown in Figures 4 and 5, rats with orchitis killed

Table 1. Morphometric study of testes from rats of nonimmunized, control, and experimental groups

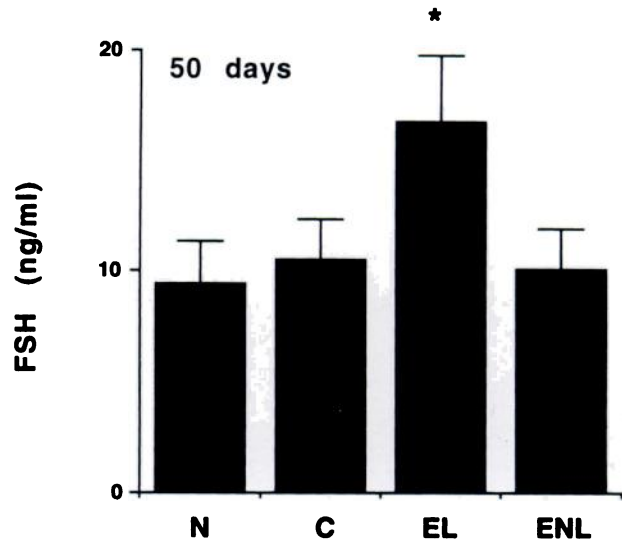
Day of sacrifice	Group	No. of Leydig cells per testis	No. of interstitial non-Leydig cells per testis	Volume density of Leydig cells per testis ( $\text{cm}^3$ )
50 days*	N	$33 \times 10^6 \pm 2,937$	$18 \times 10^6 \pm 2,566$	$0.042 \pm 0.002$
	C	$35 \times 10^6 \pm 2,400$	$19 \times 10^6 \pm 549$	$0.041 \pm 0.003$
	ENL	$31 \times 10^6 \pm 2,753$	$17 \times 10^6 \pm 743$	$0.042 \pm 0.007$
	EL	$47 \times 10^6 \pm 2,999^{\text{ac}}$	$19 \times 10^6 \pm 977$	$0.060 \pm 0.006^{\text{b}}$
80 days	N	$40 \times 10^6 \pm 1,160$	$18 \times 10^6 \pm 2,897$	$0.055 \pm 0.005$
	C	$36 \times 10^6 \pm 1,580$	$20 \times 10^6 \pm 2,263$	$0.043 \pm 0.003$
	ENL	$35 \times 10^6 \pm 2,203$	$19 \times 10^6 \pm 654$	$0.042 \pm 0.007$
	EL	$56 \times 10^6 \pm 3,233^{\text{af}}$	$24 \times 10^6 \pm 2,908^{\text{b}}$	$0.069 \pm 0.005^{\text{ab}}$

\* Days after the first immunization.

Data are expressed as mean values  $\pm$  SEM. N: nonimmunized normal rats, C: control group, ENL: experimental group without testicular lesion, EL: experimental group with orchitis. *P* values: <sup>a</sup>, 0.01, <sup>b</sup>, 0.02 (vs. 50 days control group); <sup>c</sup>, 0.01 (vs. 50 days ENL group); <sup>d</sup>, 0.002 (vs. 80 days control group); <sup>e</sup>, 0.002, <sup>f</sup>, 0.02 (vs. 80 days ENL group); <sup>g</sup>, 0.02 (vs. 80 days control group and 80 days ENL group).



**FIG. 1.** Testis sections of rats from the control group (a) and from the experimental group (b), killed at 80 days after the first immunization. In (a), few interstitial cells are present among normal seminiferous tubules. In (b), numerous lymphocytes (dark nuclei) and Leydig cells (prom-



**FIG. 2.** Serum FSH levels in rats sacrificed 50 days after the first immunization. N: nonimmunized normal rats, C: rats from the control group, EL: rats from the experimental group that developed orchitis, and ENL: rats from the experimental group without testicular lesions. Values represent the mean  $\pm$  SEM ( $n = 6-8$  rats per group). An asterisk (\*) indicates  $P < 0.05$  versus N, C, and ENL groups.

after both 50 and 80 days revealed a significant increase in T content compared to the other groups.

*In Vitro T Production*

Figures 6 and 7 show an increase in the *in vitro* basal T production in rats from the experimental group at both 50 and 80 days, compared to normal and control groups. Moreover, rats with orchitis released a higher amount of basal T compared to rats from the experimental group without lesions. In addition, all rats with testicular lesion exhibited enhancement in stimulated T production.

**Discussion**

The endocrinological profile of EAO has not been sufficiently explored. Tung et al (1984) described a decrease in serum LH and T in the dark mink with primary infertility due to an abnormal hypothalamic function. They also suggested a possible association between abnormal hormone regulation of the testis and autoimmune orchitis in the dark mink with secondary infertility (Tung and Mahi-Brown, 1990).

In the present study, we evaluated serum gonadotropins and *in vivo* and *in vitro* testicular steroidogenesis in rats with EAO. No significant hormonal changes were detected

inent nuclei with distinct chromatin) are seen among aspermatogenic seminiferous tubules. HE;  $\times 390$ .

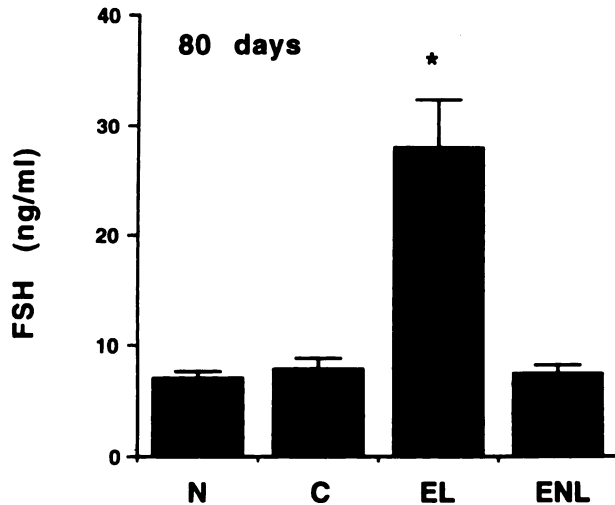


FIG. 3. Serum FSH levels in rats sacrificed 80 days after the first immunization. N: nonimmunized normal rats, C: rats from the control group, EL: rats from the experimental group that developed orchitis, and ENL: rats from the experimental group without testicular lesions. Values represent the mean  $\pm$  SEM ( $n = 6-8$  rats per group). An asterisk (\*) indicates  $P < 0.005$  versus N, C, and ENL groups.

in rats immunized with saline, CFA, and Bp (control group) compared to nonimmunized normal rats. A reduction in serum LH and T in rats injected with CFA has been described (Clemens and Bruot, 1989) in a model of arthritis, but different experimental conditions were used. We detected an increase in serum FSH in rats with orchitis, with a maximum at 80 days, at which time there was a severe histopathologic testicular lesion. A similar increase has been observed in testes with tubular damage induced by irradiation, cryptorchidism, and hydroxyurea

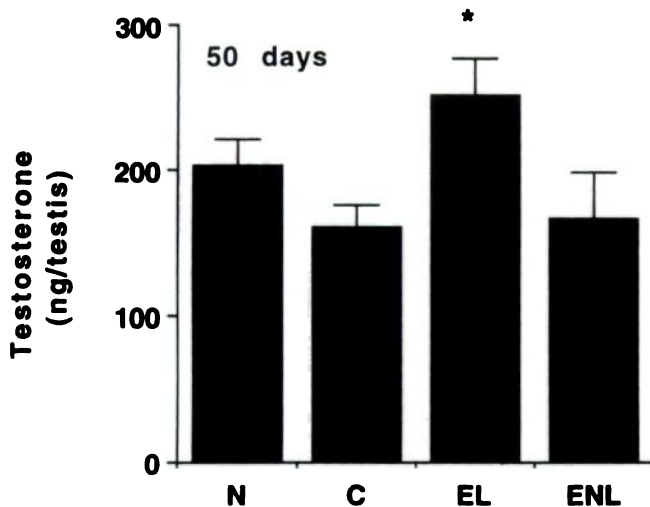


FIG. 4. Testicular T content in rats sacrificed 50 days after the first immunization. N: nonimmunized normal rats, C: rats from the control group, EL: rats from the experimental group that developed orchitis, and ENL: rats from the experimental group without testicular lesions. Values represent the mean  $\pm$  SEM ( $n = 6-8$  rats per group). An asterisk (\*) indicates  $P < 0.05$  versus N, C, and ENL groups.

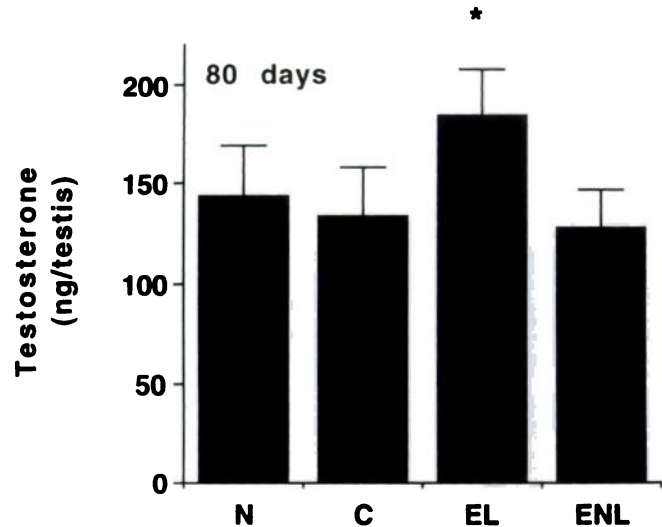


FIG. 5. Testicular T content in rats sacrificed 80 days after the first immunization. N: nonimmunized normal rats, C: rats from the control group, EL: rats from the experimental group that developed orchitis, and ENL: rats from the experimental group without testicular lesion. Values represent the mean  $\pm$  SEM ( $n = 6-8$  rats per group). An asterisk (\*) indicates  $P < 0.05$  versus C and ENL groups.

treatment, suggesting decreased inhibin production (Saez et al, 1987; Sharpe, 1990). It is particularly interesting that rats with EAO revealed a significant increase in the T content and especially in the *in vitro* basal or hCG-stimulated T release.

Because these observations were concomitant with normal LH levels, we may infer that local control mechanisms are involved. The increase in T production detected in rats from the ENL group is another phenomenon that

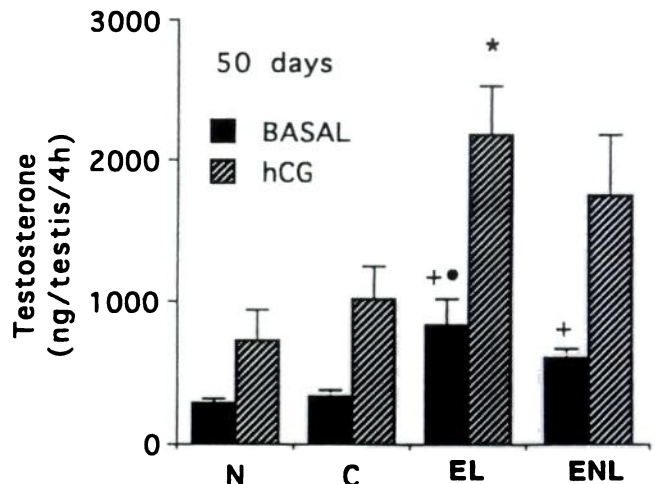


FIG. 6. *In vitro* T production in rats sacrificed 50 days after the first immunization. N: nonimmunized normal rats, C: rats from the control group, EL: rats from the experimental group that developed orchitis, and ENL: rats from the experimental group that did not develop testicular lesions. Values represent the mean  $\pm$  SEM of six flasks per point. Symbols, as follow, indicate probabilities: +,  $P < 0.05$  versus N and C groups; \*,  $P < 0.05$  versus N and C groups.

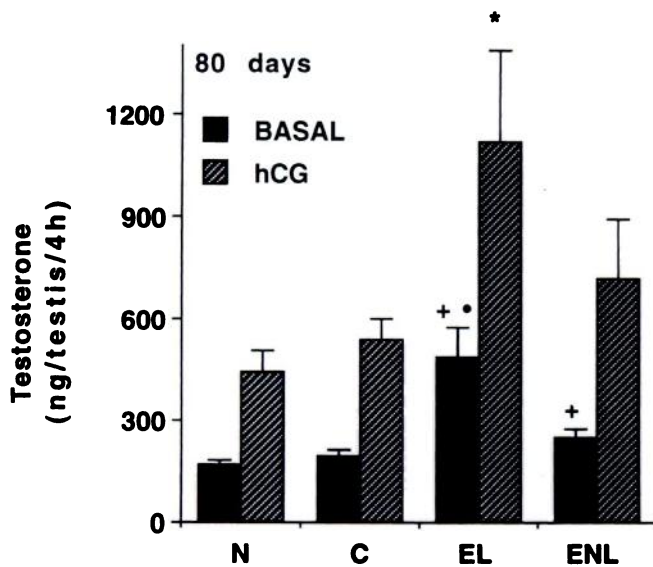


FIG. 7. *In vitro* T production in rats sacrificed 80 days after the first immunization. N: nonimmunized normal rats, C: rats from the control group, EL: rats from the experimental group that developed orchitis, and ENL: rats from the experimental group that did not develop testicular lesions. Values represent the mean  $\pm$  SEM of six flasks per point. Symbols, as follow, indicate probabilities: +,  $P < 0.05$  versus N and C groups; and \*,  $P < 0.05$  versus N, C, and ENL groups.

differentiates this group from control rats. We have previously demonstrated that, despite the lack of testicular damage, rats from the ENL group develop a humoral immune response to spermatogenic antigens and exhibit different proportions of lymphocyte subsets in the lymph nodes and in the testis (Doncel et al, 1989; Doncel and Lustig, 1991; Lustig et al, 1993). Levels of serum T in rats with EAO did not show major changes compared to control rats, except for a slight decrease at 50 days. This could be due to a lower *in vivo* T release from the testes of these animals. In fact, these data correlate with the highest increase in T content, observed at 50 days. We are not able to elucidate whether the lack of an increase in serum T depends on alterations in the blood flow of damaged testes or on the metabolic clearance of T. Our results show that the significant increase in the *in vitro* T production in rats with orchitis is concomitant with an increase in the number of Leydig cells. However, increased steroidogenesis per Leydig cell cannot be ruled out. Hedger and Eddy (1990) demonstrated *in vitro* that an increase in the Leydig cell density causes a significant enhancement in hCG-stimulated T secretion per cell; these findings suggest that if this occurs *in vivo*, an autocrine phenomenon could be involved.

In different experimental models, the induction of seminiferous tubule damage results in hyperplasia and hypertrophy of Leydig cells. A significant increase in the number of Leydig cells, concomitant with functional hyperactivity, has been observed both in testes with deple-

tion of spermatogenic cells induced by cyproterone acetate (Aoki and Fawcett, 1978) and in testes of men with germ cell tumors (Lauke et al, 1989). Because Leydig cell hyperplasia has only been observed in the vicinity of damaged seminiferous tubules, a local control mechanism has been suggested. Ojeifo et al (1990) demonstrated that Sertoli cells secreted proteins (SCSP) that stimulate DNA synthesis of purified rat Leydig cells *in vitro*. The secretion of this factor is stimulated independently by FSH or T, both of which are increased in the present study in rats with induced orchitis. According to the above authors, SCSP could behave as a paracrine regulator of Leydig cell proliferation.

In addition, the existence of factors secreted by Sertoli or other testicular cells that enhance Leydig cell steroidogenesis should be considered. It has been suggested that germinal cells modulate steroidogenesis via Sertoli cells (Qureshi and Sharpe, 1992). Jansz and Pomerantz (1987) reported an increase in basal and LH-stimulated T by Leydig cells incubated with the interstitial fluid from rat testes after a variety of treatments that disrupt gametogenesis. Several authors (Verhoeven and Cailleau, 1985; Papadopoulos, 1991; Murai et al, 1992) have partially characterized a 10- to 13-kDa protein isolated from Sertoli cell-conditioned medium that stimulates steroidogenesis in Leydig cells.

As we described in the Results section, numerous interstitial non-Leydig cells (mainly lymphocytes and macrophages) are present in the interstitial tissue of rats with EAO. It has been reported that macrophages are physically associated with Leydig cells and able to respond to FSH (Geierhaas et al, 1991; Hutson, 1992). It is widely accepted that the interleukin 1 (IL-1) secreted either by Sertoli cells or by interstitial macrophages could affect testicular steroidogenesis. In fact, several authors have described either an inhibition (Calkins et al, 1988; Fauser et al, 1989; Mayerhofer et al, 1992) or a stimulation (Verhoeven et al, 1988; Warren et al, 1990; Lombard-Vignon et al, 1991) of Leydig cell T production by IL-1. As suggested by several authors, the cell density used in different experimental conditions could partially explain the contradictory results. It has been demonstrated that culture media conditioned by testicular macrophages can stimulate T production by Leydig cells *in vitro* (Yee and Hutson, 1985).

Moreover, Bergh et al (1992) reported that testicular T was reduced in rat testes depleted of macrophages by local injection of liposome-entrapped dichloromethylene diphosphonate. Lombard-Vignon et al (1991) demonstrated that steroid production is greatly increased in Leydig cells treated with macrophage-conditioned media obtained from lipopolysaccharide (LPS)-treated testicular macrophages. This effect was not observed with unstimulated testicular or peritoneal macrophages. In our experimental

model, present and previous data (Lustig et al, 1993) show a significant increase in the number of testicular macrophages in rats with orchitis. Because an inflammatory process is concomitant with tubular damage, it is highly probable that these macrophages are in fact activated, in a manner similar to the macrophages used in the co-culture experiments of Lombard-Vignon et al (1991).

In conclusion, the present study describes the hormonal profile of adult rats with autoimmune orchitis induced by active immunization with TH. An increase in serum FSH, without changes in LH, concomitant with an increase in testicular T content and *in vitro* T production, strongly suggests a local control mechanism of Leydig cell function. Factors originating in testicular macrophages and/or in cells of damaged seminiferous tubules are probably involved in the increased steroidogenesis observed in rats with EAO.

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