The Testicular Effects of Tumor Necrosis Factor

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Tumor necrosis factor (TNF) is a cytokine that mediates many of the metabolic responses after endotoxemia, septicemia, and tissue injury. The effect of TNF on testicular function was determined in a series of studies in which rhTNF (0, 2, and 4 × 10^5 units/kg/24 hours) was administered by continuous infusion to male Wistar rats maintained on total parenteral nutrition adequate for growing rats. Testicular weight and histology, and plasma luteinizing hormonr (LH), follicle-stimulating hormone (FSH), and testosterone levels were determined at 1, 3, and 6 days. Testicular weight decreased within 24 hours and this was associated with a fall in plasma testosterone and increased LH and FSH levels. These changes persisted for 6 days, indicating a loss of testosterone-mediated negative feedback on gonadotropin release. Histologic examination demonstrated significant damage to the germ cells in the adluminal compartment of the seminiferous epithelium; extensive exfoliation of spermatocytes and spermatids occurred at day six. However the primary spermatogonia in the basal compartment were relatively spared. Damage to the seminiferous epithelium at earlier times was noted in some tubules. The decrease in testosterone concentration and increase in gonadotropin levels suggest that TNF interferes with Leydig cell function. Germ cell damage may be a direct effect of TNF on these cells or may occur through secondary mechanisms involving Leydig or Sertoli cell dysfunction.

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PHYSICAL STRESS, INJURY, and illness stimulate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the elaboration of glucocorticoids. However with stress the hypothalamic-pituitary-gonadal axis is also affected and plasma testosterone decreases after prolonged immobilization stress,[1] surgery under general anaesthesia,[2] and burn trauma.[3,4] The mechanisms that produce these effects are unclear but could occur through two major points of regulation: first glucocorticoids or other factors could inhibit testicular testosterone production, thus causing primary hypogonadism;[5] alternatively the stress response could affect hypothalamic-pituitary function and thus inhibit the release of luteinizing hormone (LH).[6]

Many of the pathophysiologic events that occur during critical illnesses are due to the effects of cytokines, inflammatory mediators produced by cells of the reticuloendothelial system, activated lymphocytes, and other cell types. One such cytokine, tumor necrosis factor (TNF), is a major mediator of septicemia and endotoxemia, causes stimulation of the HPA axis and has significant effects on the metabolism of trace minerals, carbohydrates, lipids, and proteins.[7] While studying the effects of chronic TNF administration on male rats, we noticed marked testicular atrophy. Subsequent studies demonstrated specific effects of TNF on testicular structure and function. Specifically TNF decreases plasma testosterone and causes disruption of the germ cell epithelium. These changes may have specific effects on reproductive function during critical illness. In addition the decrease in testosterone may contribute to the catabolic responses associated with critical illnesses.

Materials and Methods

Animal Preparation

Eighty male Wistar rats weighing 175 to 200 g were obtained from Charles River Laboratories (Wilmington, MA) and acclimatized to standard laboratory conditions for 5 days. They had free access to Purina™ rat chow (Purina Mills, Inc., St. Louis, MO) and water, and were housed individually in wire-bottomed metabolic cages (Lab

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Products, Inc., Maywood, NJ) in a constant-temperature environment with a 12-hour light/dark cycle (light cycle, 7:00 A.M. to 7:00 P.M.). Before each study they were randomly allocated into three treatment groups and animals from each group were simultaneously studied.

On day 0, the rats were anesthetized with sodium pentobarbital (50 mg/kg, Nembutal™, Abbott Laboratories, North Chicago, IL), weighed, the hair shaved from the interscapular and the abdominal regions, and the skin prepared with Clinidine Solution™ (The Clinipad Corp., Guilford, CT). Central venous catheters (internal diameter 0.0762 cm and external diameter 0.165 cm; Silastic™ Dow Corning Midland, MI) were inserted under sterile conditions. The catheter was secured in the external jujular vein with a double purse-string suture and tunneled subcutaneously to the interscapular region where it was threaded through a metal sheath attached to a swivel device (Instech Labs, Horsham, PA), which allowed free movement of the rat during the study period. The stainless-steel button of the metal sheath was secured to the deep fascia with sutures.

Study Protocol

After operation the animals received an intravenous infusion of 0.9% saline, 25 mL/24 hours, and the next day a standard total parenteral nutrition (TPN) solution was begun. The nutrient mixture contained adequate calories and nitrogen for growing rats (46 Kcal of nonprotein calories and 0.47 g nitrogen/rat/24 hours) and was administered at a constant infusion rate of 48 mL/24 hours.[8] Three treatment groups were studied: the control group received the TPN solution alone (TNF 0 group), and two TNF groups who received either 2 or 4×10^5 units TNF/kg rat/24 hours added to the TPN solution (TNF 2 and TNF 4 groups, rhTNF, Asachi Chemical Industry Inc., New York, NY). Previous studies have demonstrated no loss of bioactivity of TNF during 24 hours when added to the TPN solution. The rats were allowed to drink water ad libitum. Following 1, 3, and 6 days of infusion animals were killed by intraperitoneal sodium pentabarbitol (75 mg/kg) and blood was collected by cardiac puncture into heparinized tubes, centrifuged at 4000 rpm, and plasma stored at −70 C. Immediately after exsanguination the left testis was removed and weighed and the right testis was removed and processed for histologic examination.

Analytical Procedures—Histologic Preparation

Immediately after removal the right testis was diced into 1-mm cubes and placed in 3% glutaraldehyde in cacodylate buffer (0.2 m; ph 7.4) for 30 minutes. The tissue fragments were then washed three times in cacodylate buffer for 20 minutes each. The tissue was embedded in paraffin wax and stained with hematoxylin and eosin for light microscopic examination.

Hormone Assays

Testosterone, follicle-stimulating hormone (FSH), and LH were measured by radioimmunoassay (RIA). Plasma testosterone RIA was based on a modification of the method of Challis et al.[9] FSH was expressed in terms of the NIDDK rFSH-RP_2 standard and LH was expressed in terms of the NIDDK rLH-RP_3 standard. All samples were run concurrently, eliminating interassay variation. The intra-assay variation was 6.4%, 7.3%, and 9%, respectively, and the assay limits were 20 pg/mL, 300 pg/mL, and 2.5 pg/mL, respectively, for LH, FSH, and testosterone.

Statistical Methods

Results are expressed as mean ± SEM. One-way ANOVA was used for comparison of means at a single time point and two-way ANOVA was used to examine group effects with time. The Dunnett t test was used for *post hoc* comparisons when necessary. Differences were considered significant when p values were less than 0.05.

Results

Testicular Weight

Testicular weight decreased in the animals receiving TNF, in a dose-dependent fashion ($p < 0.006$). Decreased testicular weight was observed in both the low- and high-dose TNF groups in comparison to the control group. These changes occurred after 1 day of TNF infusion and persisted for the duration of the study (Fig. 1).

Hormones

Plasma testosterone decreased rapidly after 24 hours of TNF infusion ($p < 0.0005$; Fig. 1). With TNF exposure plasma testosterone levels at the 3- and 6-day periods were frequently lower than the range of assay detection. LH levels increased after 1 day of TNF infusion ($p < 0.005$) and FSH levels also increased in the TNF groups. However only the high-dose TNF significantly elevated FSH in comparison to the controls at day 6 ($p < 0.005$).

Histology

Significant destruction of the spermatogenic cells occurred with TNF infusion (Fig. 2). Extensive exfoliation of spermatogenic cells was observed in the adluminal compartment, affecting all developing spermatocytes and spermatids. Accumulation of dead cells and debris was evident and obliterated the lumen in many sections. These changes appeared to be dependent on TNF dose. The

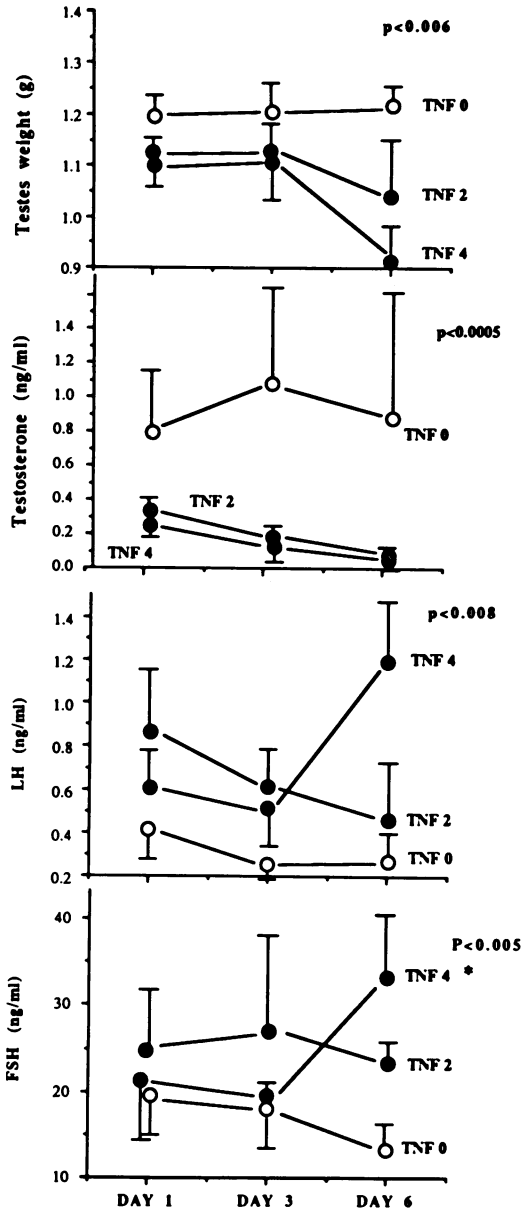


FIG. 1. Effect of TNF on the pituitary-testicular axis. Testis weight and plasma testosterone, LH and FSH in control saline-infused (TNF 0), and low and high dose TNF-infused groups (TNF 2 and TNF 4), at 1, 3, and 6 days. Results are expressed as mean ± SEM. p values represent group effects over time and the asterisk refers to a significant TNF 4 s TNF 0 group effect at day 6.

majority of tubules were affected after 6 days of TNF infusion. High-dose TNF eliminated all of the testicular sperm and the majority of spermatids at all stages of differentiation. At earlier times identical effects were observed in 5% to 10% of tubules. Spermatogonia found in the basal compartment below the Sertoli cell tight junctions appeared resistent to the destructive effects of TNF. However increased vacuolization in the basal epithelial compartment surrounding the spermatogonia was noted at day 6 in animals receiving the high-dose TNF infusion.

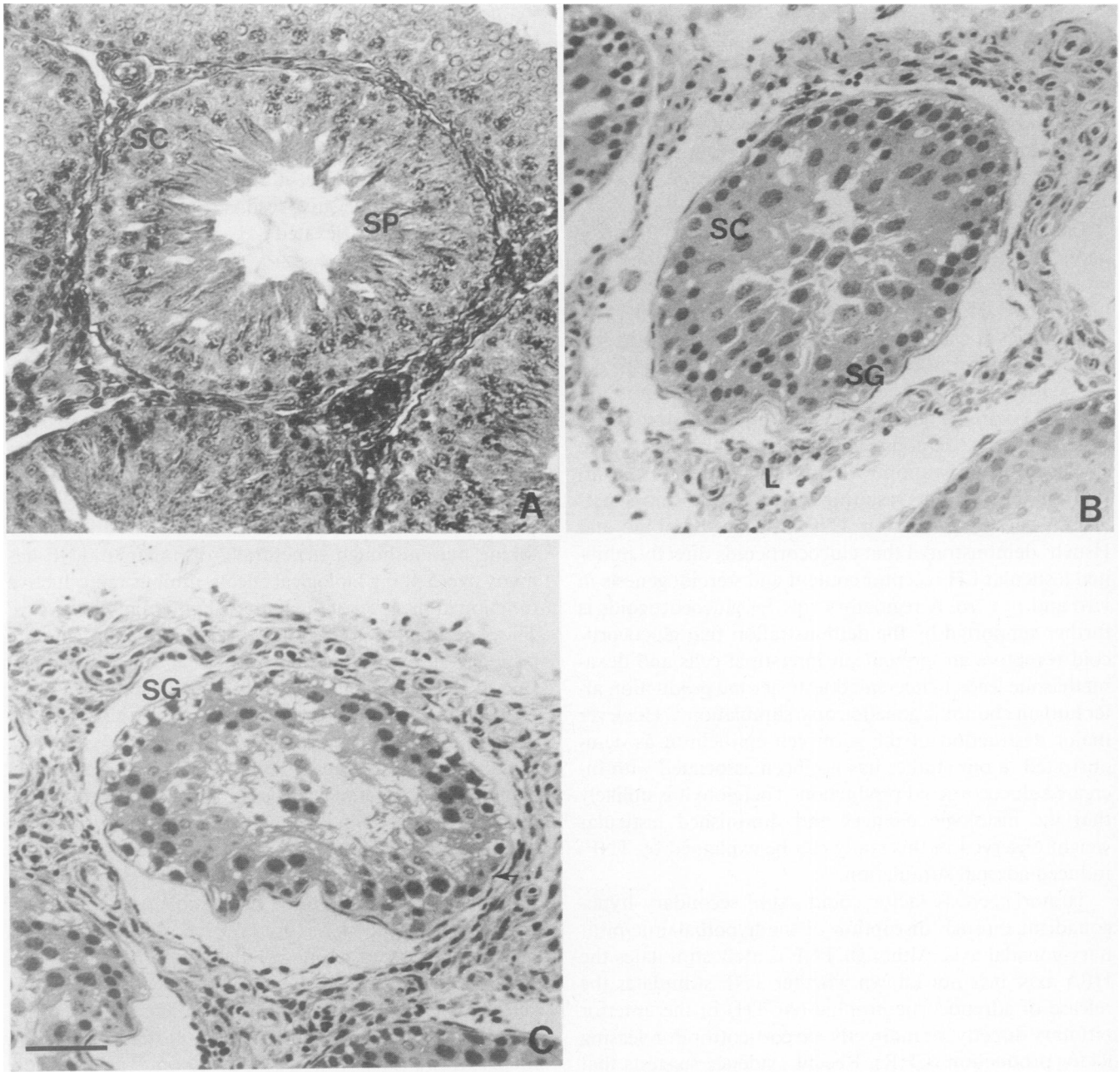
Although Sertoli and Leydig cell morphology are difficult to assess with the light microscope, counts of Sertoli cell nuclei did not appear to be decreased and no significant alterations were observed in Leydig cell numbers in the TNF-infused animals.

Discussion

Tumor necrosis factor has significant detrimental effects on testicular structure and function. The primary spermatogonia in the basal tubular compartment below the Sertoli tight junctions were relatively resistent to TNF effects, whereas spermatocytes and maturing spermatids within the adluminal compartment above the tight junctions were severely affected. Sertoli cell tight junctions exclude molecules exceeding approximately 10,000 daltons and probably exclude TNF (17,000 daltons molecular weight) from the adluminal compartment. Thus damage to the late germ cells may have occurred, due to increased sensitivity of these cells to TNF, after TNF passage through the supporting Sertoli cells. Alternatively late germ cell damage may be secondary to TNF-induced changes in Sertoli cell function.

No obvious light microscopic changes were observed in either the Leydig or Sertoli cell populations. Leydig cell number appeared unaltered, despite the rapid decrease in testosterone levels that occurred within 24 hours of TNF exposure. To detect fine details of Sertoi cell structure, ultrastructural analysis must be used, particularly to examine the integrity of the inter-Sertoli cell tight junctions that are located immediately above the basally located spermatogonia. Structural maintenance of these occluding junctions would likely prevent access of TNF to differentiating spermatocytes and spermatids. Intact seminiferous tight junctions, therefore, would strongly implicate a direct effect of TNF on Sertoli cells or, less likely, the passage of this cytokine through the Sertoli cells with resultant damage to germ cells located adluminally. Preliminary electron microscopic observations indicate that at least some of these tight junctions remained intact after TNF treatment (unpublished observations). However precise assessment of the integrity of these tight junctions would require testicular perfusion with tracer molecules such as lanthanum or freeze-fracture analysis of the seminiferous epithelium after TNF exposure.

Studies in hypophysectomized animals indicated that disruption of the pituitary-testicular axis can lead to germ cell atrophy.[10,11] It is possible, therefore, that the decreased testosterone levels observed in these experiments gave rise to destruction of developing sperm. However the temporal sequence of histologic changes in this study occurred much more rapidly than those described in hypophysectomized animals.[10,11] Furthermore studies in hypophysectomized animals demonstrated comparable destruction of both



FIGS. 2A–C. Histological sections of rat testis following TNF infusion. (A) Saline-infused control testis at 6 days. Additional samples, exposed to TNF for 6 days, illustrate effects caused by TNF 2 (B) and TNF 4 (C), respectively (magnification ×360, reference bar = 50μ). Extensive exfoliation of spermatogenic cells in the adluminal compartment of the seminiferous tubules is evident. Cells affected include spermatocytes (CS), with few spermatids (SP) surviving TNF exposure. Spermatogonia (SG), in the basal compartment, appear less affected, although increased vacuolization surrounding these cells is noted in TNF 4 samples. Leydig cells (L) do not seem to be significantly affected at this level of analysis. Sertoli cell nuclei (arrow head) also are not diminished.

primary spermatogonia and late germ cells. In our studies the predominant TNF effect occurred almost exclusively on the late germ cells. It is likely that the adluminal destruction of the spermatocyte and spermatid populations is due to the direct effects of TNF and not secondary to changes in circulating androgen and gonadotropin levels.

Tumor necrosis factor production is stimulated by endotoxin; therefore administration of endotoxin should induce similar changes in testicular structure and function.[12] In fact Tulassay and colleagues[13] have demonstrated that a single intraperitoneal injection of endotoxin significantly decreased testis weight and inhibited maturation of spermatocytes. These investigators found that this effect was reversible and that the primary spermato-

gonia were also relatively resistence. Christeff and coworkers[14] demonstrated that a sublethal intravenous injection of endotoxin increased plasma estrogen and decreased testosterone in male rats; these responses were of rapid onset with maximal effect within 2 hours. Because the effects after endotoxin administration are similar to our observations, it is likely that they are mediated by TNF elaboration.

Increased glucocorticoids may cause primary hypogonadism and diminish androgen production. Tumor necrosis factor acutely elevates corticorticoid levels;[15,16] hence TNF effects on the testes could be secondary to glucocorticoid elaboration. General anesthesia,[2] burn injury,[3,4,17] and immobilization stress[1] all lead to both decreased testicular and adrenal androgen production and increased adrenal glucocorticoid output. Dolecek and colleagues[17] also demonstrated that plasma LH levels were elevated in combination with diminished testosterone after burn trauma, thus resulting in a similar hormone pattern to that observed after TNF infusion. Bambino and Hsueh[5] demonstrated that glucocorticoids directly inhibited testicular LH receptor content and steroidogenesis *in vivo* and *in vitro*. A regulatory role for glucocorticoids is further supported by the demonstration that glucocorticoid receptors are present on interstitial cells and dexamethasone leads to decreased testosterone production after human chorionic gonadotropic stimulation.[18] However major destruction of the germ cell epithelium as demonstrated in our studies has not been associated with increased glucocorticoid production. Therefore it is unlikely that the histologic changes and diminished testicular weight observed in this study can be explained by TNF-induced adrenal stimulation.

Tumor necrosis factor could cause secondary hypogonadism through disruption of the hypothalamic-pituitary-gonadal axis. Although TNF acutely stimulates the HPA axis, it is not known whether TNF stimulates the release of adrenocorticotrophic (ACTH) in the anterior pituitary directly, or indirectly *via* corticotropin-releasing factor production (CHR). Recent evidence suggests that TNF (or even an unknown intermediate) may directly stimulate pituitary ACTH release[19,20] without stimulation of the central nervous system.[21] It is not known whether CRH and β-endorphin (βEP), two central mediators of the stress response known to influence the pituitary release of LH, are affected by TNF. Rivier and colleagues[22] have demonstrated that increased CRH inhibited LH release, most likely by inhibiting gonadotropic-releasing hormone (GnRH) release into the portal circulation. This effect is seen in adrenalectomized animals.[22] Opiate agonists also have been implicated in the suppression of LH release.[23] Interestingly bacteremia, which is a potent stimulant of βEP, has also been shown to decrease LH release.[24] Gindoff and colleagues[25] and Ferin[26] have suggested that CRH-induced inhibition of LH release in the primate is regulated by endogenous opioid peptides. It is not known whether TNF increases βEP levels in either the hypothalamus or the pituitary, although it is of interest that βEP and ACTH in the pituitary are both derived from a common precursor, pro-opiomelanocortin (POMC).[27] However, if these mediators of the stress response lead to inhibition of LH release, it appears that the loss of testosterone-induced negative feedback overcomes this effect and gives rise to the elevated LH and FSH levels observed in this study.

The messenger RNA's encoding CRH, POMC, and several opioid peptides, including βEP, are also found locally in rat epididymis, Leydig, and germ cells.[28–30] Although the function of these peptides is not clear, they may suppress testosterone production by the Leydig cell and regulate Sertoli cell growth.[31] A regulatory role for POMC peptides in the testes is supported by the observation that CRH stimulates β-endorphin secretion by rat Leydig cells in culture.[32] Recently Rivier and Vale[33] have demonstrated that Interleukin-1 alpha (Il-1), another cytokine that, although structurally disimilar to TNF has many overlapping biological effects, inhibits reproductive function at the level of the hypothalamus and the gonads. They speculate that because Il-1 stimulates POMC in the pituitary it may also have similar effects on gonadal POMC. Likewise TNF may stimulate gonadal POMC and exert some of the effects demonstrated in this study *via* opioid peptide release.

Spratt and colleagues[34] demonstrated that plasma LH can remain normal or increase in association with falling testosterone levels in critically ill men, and have suggested that hypoandrogenemia may be due to both gonadal and hypothalamic-pituitary effects that may be dissociated in time. Our results seem to be compatible with these findings. Apparently TNF has direct effects on the testis, causing decreased testosterone production and germ cell degeneration. Testosterone is diminished within 24 hours, yet LH and FSH levels only increase after 6 days of infusion, which is unlike gonadectomy in male rats, which increases LH levels within 8 to 24 hours.[35,36] These low testosterone levels may attenuate reproductive behavior during illness associated with cytokinemia. In addition the fall in androgens may mobilize carcass substrate to be used by visceral organs to facilitate metabolic host responses and tissue repair.

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