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Testicular Dysfunction in the Adjuvant-Induced Arthritic Rat

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Adjuvant-induced arthritis, an autoimmune disease similar to rheumatoid arthritis, was used to investigate possible mechanisms of immune system modulation of the reproductive system. This laboratory previously reported that arthritic male rats have reduced serum testosterone and elevated serum LH concentrations. In the experiments described here, serum prolactin levels were not significantly different in arthritic animals compared with non-injected control animals. Neither reduced food consumption of arthritic rats nor the injection vehicle appear to cause a reduction of serum testosterone. Serum corticosterone was significantly elevated in the arthritic group compared with both the non-injected or the vehicle-injected control animals. Testicular cells from arthritic animals secrete significantly less testosterone *in vitro* compared with cells from non-injected control animals, both basally and in response to dbcAMP and hCG. In summary, the reduced serum testosterone of arthritic animals appears to be the result of a testicular dysfunction.

Key words: Testosterone, corticosterone, adjuvant arthritis, testicular function, immune-reproductive axis.

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It is generally accepted that sex hormones exert a regulatory influence over the immune system (Grossman, 1984). The immune system also may influence reproductive function. Until recently, most knowledge about the relationship between the reproductive system and the immune system was inferred from clinical studies. A renewed interest in the immune and reproductive systems, however, has resulted in several recent reports of a direct interaction between immune cells and the gonads. Yee and Hutson (1985) reported that testicular-derived macrophages secrete a substance that stimulates testicular steroidogenesis. Hiriart and Romano (1986) reported that a thymic factor inhibited the binding of hCG to Leydig cell membranes. The factor also inhibited hCG-stimulated testosterone secretion by Leydig cells in short-term incubation (Pedernera et al, 1986). Recently, Verhoeven et al (1988) reported that interleukin 1 (IL-1) stimulated testosterone secretion by cultured immature rat Leydig cells in the absence of LH or in the presence of low doses of LH. In the presence of higher concentrations of LH,

however, IL-1 blocked the 17-20 desmolase enzyme and hence reduced testosterone synthesis.

A recent report by Bruot and Clemens (1987) that male rats with adjuvant-induced arthritis have reduced serum levels of testosterone suggests the possibility that the adjuvant-induced arthritic rat may be a good model in which to study the influence of the immune system on the reproductive system. The adjuvant-induced arthritic rat is commonly used to study rheumatoid arthritis (Billingham and Davies, 1979). Arthritis is induced by the injection of an oil containing heat-killed mycobacterium and results in an erosion of articular cartilage and periarticular bone (Taurog, 1987). Arthritis develops because the immune system is unable to distinguish between the mycobacterial antigens and native cartilage proteoglycans (van Eden et al, 1985). It is possible, however, that other factors independent of the immunological aspect of the arthritis may have influenced serum testosterone levels in the arthritic rats. This study was undertaken to examine some of these possibilities and to compare steroidogenesis by dispersed testicular cells from arthritic and non-arthritic animals.

Materials and Methods

Animals

Male Lewis rats (Charles Rivers, Inc., Wilmington, MA) between 55 and 60 days of age were housed under controlled lighting (12L:12D) conditions in a temperature (21 ± 2C) controlled room. In the first experiment, animals were randomly assigned to either a control (non-injected; n = 10) or arthritic (injected with Freund's Complete Adjuvant; n = 10) In the second experiment, animals were randomly assigned to either a non-injected control group (n = 10), a group injected with Freund's Incomplete Adjuvant (n = 10), or a group made arthritic by injection with Freund's Complete Adjuvant (n = 10). In the third experiment, non-injected control animals were assigned to either a restricted food intake group (n = 10) or a group fed *ad libitum* (n = 10). In the fourth experiment, animals were assigned to one of two non-injected control groups (n = 6/group) or to one of two arthritic groups (n = 6/group).

Induction of Arthritis

On Day One of the experiment, rats were anesthetized and injected with 0.1 ml of Freund's Incomplete Adjuvant (Difco, Detroit, MI) or 0.1 ml of Freund's Complete Adjuvant (Freund's Incomplete Adjuvant containing 10 mg/ml *Mycobacterium butyricum*) into the right hind foot pad (Bruot and Clemens, 1987). All animals were housed in shoe box-type cages on wood shavings to minimize discomfort. Trunk blood was collected on day 21 or 22 post-injection by rapid decapitation. Body and right testis weights were determined. Left and right hind paw volumes were also measured with a mercury plethysmograph to determine the severity of arthritis.

Testicular Dispersion and Incubation

The testes were excised, decapsulated in medium 199, and mechanically dispersed in a shaking water bath for 15 min. The tubules were then allowed to sediment and the supernatant was centrifuged at 1000 rpm for 5 min. to obtain the dispersed cells. Cell viability, as determined by trypan blue dye exclusion (Tennant, 1964) averaged 96% for cells from the arthritic rats and 90% for cells from the control animals. Approximately 6×10^6 testicular cells were incubated in a shaking water bath (60 cycles/min) at 37 C in 2 ml of M199-0.1% BSA for 4 h. In an independent series of experiments, testosterone secretion by such testicular preparations was found to be linear for 4 h. Similar preparations were also found to be 22.7% ± 2.89 Leydig cells by 3β-hydroxysteroid dehydrogenase staining. Some cells from one of the non-injected control groups and one of the arthritic groups were incubated in a medium supplemented with dibutyryl cAMP. Similar cell preparations were incubated with human chorionic gonadotropin (hCG). After each incubation, cells were separated from the medium by centrifugation and the medium was stored frozen at −20 C until analyzed for testosterone.

Hormone Analysis

Serum testosterone was measured directly by radioimmunoassay (RIA) after extraction with diethyl ether. The details of this assay have been described elsewhere (Lee et al, 1986; Bruot and Clemens, 1987). A preliminary experiment showed a good correlation between the testosterone in incubation media samples analyzed after ether extraction and without extraction. Therefore, testosterone in the incubation medium was analyzed without extraction.

Corticosterone was measured directly from serum by RIA without extraction as described by Radioassay Systems Laboratories, Inc. (RSL technical bulletin 10/85; Carson, California). Briefly, standards and unknowns were heated to 98C for 10 min to denature corticosterone binding proteins. After cooling to room temperature, an aliquot of anti-corticosterone (RSL No. 1470) and [^{3}H]-corticosterone were added to each tube. The tubes were then incubated for 1 h at 4C. Antibody-bound corticosterone was separated from free corticosterone by the addition of charcoal-dextran solution to each tube, followed by centrifugation. The corticosterone antisera cross reacted 33% with progesterone, 6% with 20-dihydroprogesterone, 5% with cortisol, and less than 3% with all other steroids tested. All samples were analyzed in the same assay. The intraassay coefficient of variability was 7%.

Prolactin concentrations were analyzed by RIA using a kit supplied by the National Pituitary Agency, NIAMMD. Results were expressed in terms of rat prolactin RP-3. Details of this assay have been published elsewhere (Lee et al, 1986).

Statistical Analysis

In the first and third experiments, the results were analyzed using student's t-test. Results from the second experiment were tested for homogeneity of variance using the Bartlett's test and log transformed if nonhomogeneous. For ease of discussion, only nontransformed results were described. Significant differences were determined using one-way ANOVA and Tukey's test. Pearson's product-moment correlation analysis was used to determine relationships among variables. In the fourth experiment, signficant differences were determined by two-way ANOVA and Newman-Keul's test. All differences were considered significant if the P value was less than 0.05. The results are expressed as the mean ± SEM.

Results

Experiment I

Serum prolactin levels in the arthritic rats were not significantly different than in the non-injected control animals. Serum concentrations in the arthritic rats were 8.5 ± 1.5 ng/ml (n = 10) compared to 6.1 ± 0.9 ng/ml (n = 10) in the control animals. In contrast, serum concentrations of testosterone in the arthritic rats (1.2 ± 0.2 ng/ml, n = 10) were significantly less than in the control animals (2.7 ± 0.3 ng/ml, n = 10).

Experiment II

The effect of Freund's Incomplete Adjuvant on paw volume, testis weight, and serum concentrations of testosterone and corticosterone were examined in this experiment. The injection of Freund's Incomplete Adjuvant signficantly increased paw volume in the injected paw when compared to that in non-injected animals (Table 1). Paw volume of the injected paw in Freund's Complete Adjuvant-injected animals, however, was significantly greater than that in either of the other groups. As expected, Freund's Incomplete Adjuvant had no effect on contralateral paw volume. Furthermore, the treatments had no significant effect on testis weight. There were 10 animals in each group.

TABLE 1. Effect of Freund's Incomplete and Complete Adjuvant on Body Weight, Testis Weight, and Paw Volume

	Weight (g)		Paw Volume (ml)	
Treatment	Body	Testis	Left	Right
Non-injected	293±4[a]	1.41±0.07	0.97±0.04[a]	1.02±0.03[a]
Incomplete	286±4[a]	1.37±0.08	0.94±0.02[a]	1.45±0.05[b]
Complete	207±4[b]	1.34±0.07	1.62±0.05[b]	3.55±0.09[c]

Values are expressed as mean ± SEM. Means within a column with different superscripts are significantly (P < 0.05) different.

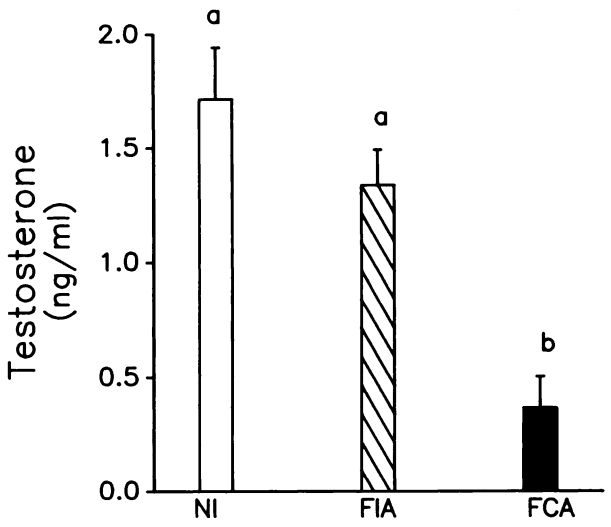

Fig. 1. Serum testosterone concentrations in non-injected (NI) control rats, Freund's Incomplete Adjuvant (FIA)-injected rats, and Freund's Complete Adjuvant (FCA)-induced arthritic rats from Experiment II. Means with different letters are significantly (P < 0.05) different. The results are expressed as mean ± SEM of 10 rats/group.

Serum concentrations of testosterone in the Freund's Incomplete Adjuvant-injected animals were not significantly different than in the non-injected control animals (Fig. 1). In contrast, testosterone levels in the Freund's Complete Adjuvant-injected arthritic rats were reduced approximately 76% and 71% when compared to the levels found in the non-injected and Freund's Incomplete Adjuvant-injected animals, respectively.

Serum corticosterone concentrations were significantly elevated by Freund's Complete Adjuvant injection (Fig. 2). Corticosterone concentrations in the Freund's Complete Adjuvant-injected rats were elevated eight-fold compared to the non-injected animals and three-fold compared to the Freund's Incomplete Adjuvant-injected animals. Serum concentrations of corticosterone in the Freund's Incomplete Adjuvant-injected rats were elevated two-fold compared to the non-injected control animals, although this difference was not significant. A negative correlation was found between the serum corticosterone and testosterone concentration. Testosterone levels were significantly lower in those animals with higher corticosterone concentrations.

Experiment III

In the previous experiment, the amount of food consumed by the arthritic rats was determined to be 12 g of rat chow/day compared to 18 g/day for

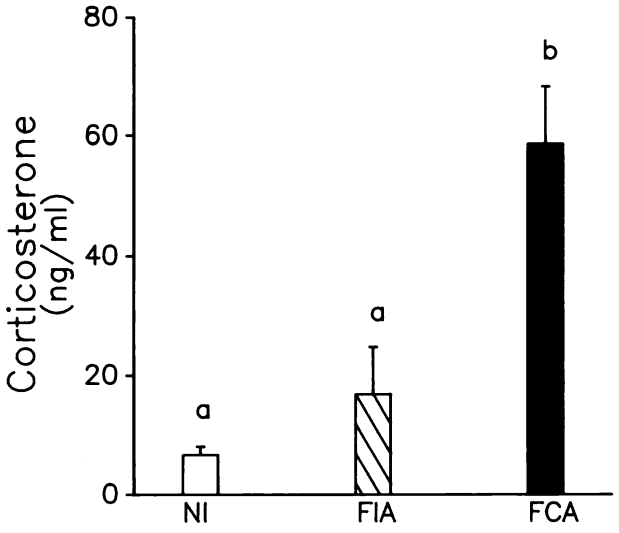

Fig. 2. Serum corticosterone concentrations in NI control rats, Freund's Incomplete Adjuvant-injected rats, and Freund's Complete Adjuvant-induced arthritic rats from Experiment II. Means with different letters are significantly ($P < 0.05$) different. The results are expressed as mean ± SEM of 10 rats/group.

the non-injected control animals. In this experiment the rats were divided into two groups. One group of rats was given 12 g rat chow/day while a second group was fed *ad libitum*. Predictably, those rats on the reduced diet weighed significantly less (208 ± 3 g vs. 290 ± 9 g) at the end of the experiment. Serum testosterone concentration in non-injected rats given 12 g of chow/day was compared with the levels found in non-injected animals fed *ad libitum*. Serum testosterone concentrations in animals fed *ad libitum* were 1.0 ± 0.3 ng/ml (n = 10) compared with 1.0 ± 0.1 ng/ml (n = 10) in the animals restricted to 12 g of rat chow/day.

Experiment IV

Testicular cells from arthritic rats secreted significantly less (60-70%) testosterone than cells from non-arthritic animals (Fig. 3). The testicular cell preparations from both groups were responsive to dbcAMP and hCG stimulation. The addition of 5 mM dbcAMP stimulated a 240% increase in testosterone secretion by cells from arthritic animals compared with a 270% increase in secretion by cells from non-arthritic animals. Similarly, a 250% increase in testosterone secretion by cells from arthritic rats was observed in response to 3 mIU hCG compared with a 320% increase from testicular cells from non-arthritic animals. The amount of testosterone secreted by testicular cells from arthritic rats was at all doses of hCG or dbcAMP, however, significantly less than the amount secreted by testicular cells from non-arthritic animals.

Discussion

Several recent studies have reported reduced serum levels of testosterone in men with rheumatoid arthritis (RA). Gordon et al. (1986) reported that although men with RA had elevated levels of LH and FSH, they had significantly lower levels of serum testosterone. Prolactin and cortisol concentrations were within the normal range. More recently, Spector et al (1988) reported that although serum levels of testosterone were reduced in men with RA, this difference was not significant. They found, however, that serum levels of free testosterone were

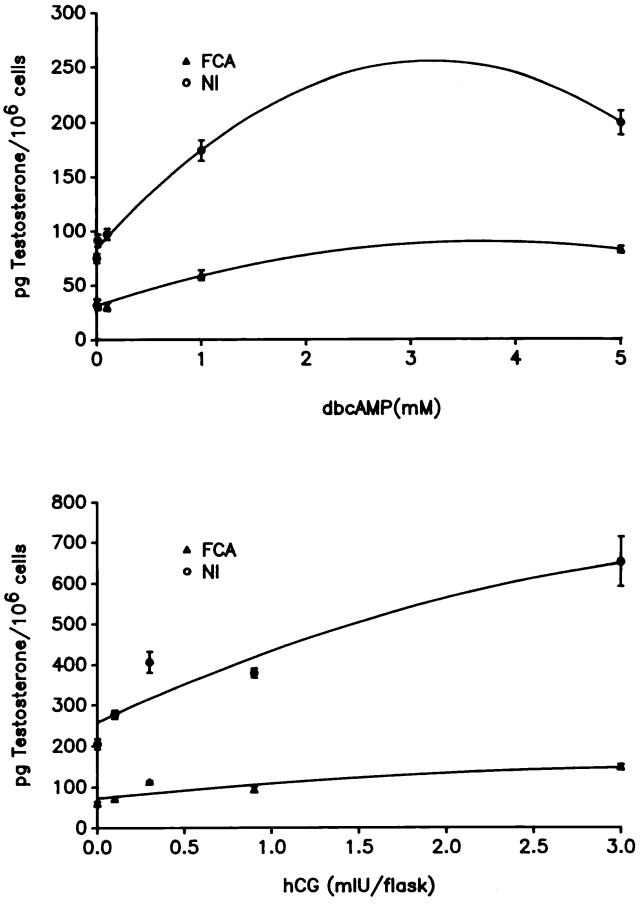

Fig. 3. Testosterone secretion by testicular cells from Freund's Complete Adjuvant-induced arthritic and non-injected control rats incubated for 4 h with dbcAMP (top) and hCG (bottom). Each point represents the mean ± SEM of 3-4 replicates. The concentrations of testosterone secreted by Freund's Complete Adjuvant-induced arthritic rats is significantly ($P < 0.05$) less than non-injected control animals at all concentrations of dbcAMP and hCG.

significantly reduced when compared to osteoarthritic men. They found no differences in LH, FSH, progesterone, androstenedione, or DHEAS. Levels of cortisol and sex hormone binding globulin were also non-significantly elevated in the men with RA. Thus, although there are some differences between these two studies, there is sufficient evidence to hypothesize that reduced serum testosterone may be a general feature of RA.

These results are similar to a recent finding from this laboratory that serum levels of testosterone were reduced in male rats with adjuvant-induced arthritis (Bruot and Clemens, 1987). It is possible, however, that adjuvant injection may affect testosterone synthesis via mechanisms unrelated or secondary to the development of the arthritis. This may be the result of an effect of the incomplete adjuvant on testosterone synthesis. The results from the second experiment in the present study clearly show that Freund's Incomplete Adjuvant and the local, nonsystemic inflammation associated with it had no effect on serum testosterone levels, even in the presence of slightly elevated corticosterone concentrations. Furthermore, it is unlikely that the reduction in serum testosterone can be attributed to autoimmune orchitis because there were no significant differences in testis weight. These results suggest that "incomplete-adjuvant alone" was not responsible for the decrease in serum testosterone.

It is also possible that reduced food consumption may have contributed to a reduction in serum testosterone in arthritic rats. Pirke and Spyra (1981) reported that starvation reduced testosterone and LH concentrations in the adult male rat. In the present experiment, however, rats given less food gained significantly less weight; yet their testosterone values were indistinguishable from animals fed *ad libitum*. Thus, these results suggest that a reduction in food consumption was not responsible for the reduction in testosterone.

Previously, this laboratory reported that animals with reduced serum levels of testosterone had elevated levels of serum LH (Bruot and Clemens, 1987). This observation suggested that the effect of adjuvant-induced arthritis may be the result of altered testosterone synthesis. The results reported in this paper are consistent with this hypothesis. Testicular cells from arthritic rats secrete significantly less testosterone than those from non-arthritic animals. The testicular cells from arthritic rats were responsive to both dbcAMP and hCG. Although the percent increase in testosterone secretion by testicular cells from both arthritic and non-arthritic rats were similar, the amount of testosterone secreted by testicular cells from control animals was always significantly greater than from arthritic animals.

Thus, it is possible that the reduction in serum testosterone was the result of a loss and/or uncoupling of LH receptors in the arthritic animals. The mechanism whereby LH receptor may be reduced or uncoupled is not known; however, macrophage production of IL-1 is known to be elevated in arthritic rats (Johnson et al, 1986). Furthermore, IL-1 production has been shown to inhibit LH receptor formation in cultured rat granulosa cells (Gottschall et al, 1988). The fact that the adult rat testis contains 15–20% macrophages (Themmen et al, 1987) suggests the possibility that IL-1 production by activated testicular macrophages may have a similar effect on the Leydig cells in the arthritic rat.

Finally, it is possible that the elevated levels of corticosterone may have been responsible for the reduction in testosterone. Elevated levels of corticosterone may indirectly decrease testicular steroidogenesis by decreasing the serum levels of LH and/or PRL (Baldwin and Sawyer, 1974; Harm et al, 1975). It is unlikely that decreased LH was involved because serum levels of LH have been reported to be elevated in the adjuvant-induced arthritic rat (Bruot and Clemens, 1987). Several reports have suggested a role for prolactin in the inhibitation of Leydig cell steroidogenesis *in vitro* (Papadopoulos et al, 1986; Barkey et al, 1987). However, prolactin is unlikely to be the cause of reduced testicular steroidogenesis by arthritic testicular cells because in this study, PRL levels in the arthritic rats were indistinguishable from the levels in the non-arthritic animals. Alternatively, corticosterone may have directly affected testosterone synthesis. Persellin et al (1972) reported adrenal hypertrophy and elevated corticosterone in rats with adjuvant-induced arthritis. Bambino and Hsueh (1981) reported that several glucocorticoids directly inhibit testosterone synthesis. The significant correlation between testosterone and corticosterone levels in the present study opens the possibility of a direct effect of glucocorticoids on testicular steroidogenesis.

In summary, serum testosterone levels in the adjuvant-induced arthritic rat are significantly less than those found in non-arthritic animals. This reduction is not the result of an effect of the adjuvant

vehicle alone. Furthermore, it does not appear to be the result of reduced food consumption and body weight. Nor does serum prolactin appear to be involved with the reduction in serum testosterone. At this time, it is not possible, however, to rule out a direct effect of the glucocorticoids on testicular steroidogenesis. Finally, testicular cells from arthritic rats secrete less testosterone than cells from non-arthritic animals. Taken together, these results suggest that the reduction in testosterone may be the result of a direct effect of adjuvant-induced arthritis on testosterone synthesis. Additional studies will be required to determine the mechanism responsible for this reduction.

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Postdoctoral Position

A postdoctoral position to study the biochemistry and physiology of ABP is currently available. A suitable person would have a background in current biochemical and immunological techniques. Purified proteins, monoclonal and polyclonal antibodies, and the reagents needed for these studies are already on hand. Contact: Benjamin J. Danzo, PhD, Department of OB/GYN, Vanderbilt University, Nashville, TN 37232. Telephone: (615) 322-4433.