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Decreased plasma gonadotropin and testosterone levels in arthritic rats: are corticosteroids involved?

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Infectious and inflammatory diseases are often accompanied by abnormal reproductive functions, and the present working hypothesis is that proteins (called cytokines or interleukins, ILs) released by activated immune cells are at least in part responsible for these neuroendocrine changes. In order to test this hypothesis, we need paradigms of immune pathologies in which concentrations of cytokines are increased, and those of hormones of the hypothalamic-pituitary-gonadal (HPG) axis are blunted. We chose a rodent model of arthritis, adjuvant-induced arthritis (AIA), in which rats show elevated plasma IL-6 and decreased testosterone (T) concentrations. We describe here the first phase of our studies, in which we determined whether gonadotropin release was also altered, whether this change was responsible for the low T levels, and whether elevated corticosterone participated in the decreased activity of the HPG axis.

AIA is induced by the intramuscular injection of *Mycobacterium butyricum* (MBB) into the tail base of the rat, with swelling of the limbs occurring 11-12 days later. We observed significant decreases in LH and FSH secretion of castrated AIA male rats, suggesting that altered gonadotropin output was independent of the gonads. The absence of significant alterations in GnRH gene expression in the hypothalamus of AIA rats, as well as only modest declines in pituitary responsiveness to GnRH, indicate that these mechanisms are not primarily responsible for the blunted gonadotropin concentrations. Intact AIA rats exhibited a dramatic decline in T levels, but no concimitant rise in LH concentrations. The observation that gonadotropin secretion does not increase despite significantly reduced T levels suggests the presence of an unidentified defect within the GnRH neuronal circuitry that prevents the gonadotrophs to respond to decreased steroid feedback. Testicular responsiveness to hCG was significantly blunted in AIA rats, and this decrease was not reversed by acute blockade of nitric oxide formation or of prostaglandin synthesis. Interestingly, the onset of these hormonal changes preceded the appearance of symptoms (limb swelling), as well as the decrease in body weight that accompanies visible joint enlargement. On the other hand, blunted T secretion coincided with rising levels of ACTH and corticosterone. This suggested that adrenal steroids might be responsible for the decrease in LH and T values, but this hypothesis did not prove valid. Indeed, we observed that adrenalectomized AIA animals implanted with corticosterone pellets retained their low T levels. Furthermore, clamping corticosterone levels was only moderately effective in reversing the inhibitory influence of the arthritic process on LH secretion.

In the absence of significant alterations in GnRH gene expression, it is possible that low Gn levels are secondary to an abnormal pattern in GnRH pulse amplitude and/or frequency. While the decrease in plasma LH concentrations may play a role in the dramatically lowered plasma T values, it is more likely that the inability of the testes to respond to gonadotropin is of significance. While we cannot rule out the participation of perceived stress at the onset of the changes in pituitary and testicular function of AIA rats, we hypothesize that cytokines released by the inflamed tissues, an event that may well precede the appearance of overt swelling, are responsible for the activation of the HPA axis and independently, for the decreased activity of the HPG axis. The AIA model may therefore provide an experimental paradigm in which to test hypotheses related to the cross-talk between the immune system and reproductive parameters.

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Introduction

The existence of functional links between the immune system and neuroendocrine axes is well established. In animal models, inflammatory processes such as tissue injury (induced by the intramuscular injection of small volumes of turpentine), cutaneous lesions (induced by the injection of carrageean into a dorsal pouch) and arthritis [caused by the administration of *Mycobacterium butyricum* (MBB)], are all accompanied by significant increases in plasma ACTH and corticosterone levels (Neidhart & Flückiger, 1992; Sarlis *et al.*, 1992; Sternberg, 1992; Turnbull *et al.*, 1994). In both humans and experimental animals, long-term arthritis is characterized by decreased androgen secretion (Bruot & Clemens, 1987, 1989; Gordon *et al.*, 1988; Martens *et al.*, 1994). The mechanisms responsible for this altered testicular activity remain poorly understood, though they do not appear to directly involve LH (Bruot & Clemens, 1989). The fact that low testosterone (T) levels have only been reported after a protracted course of the disease, suggests the possibility that blunted testicular activity may have been at least in part induced by the stress of pain and discomfort (Millan *et al.*, 1986). Indeed hormones released by stress, in particular glucocorticoids (GC), are known to inhibit T release (Bambino & Hsueh, 1981; Lopez-Calderon *et al.*, 1991; Orr & Mann, 1992). The increased activity of the hypothalamic-pituitary-adrenal (HPA) axis of arthritic rats (Harbuz *et al.*, 1992; Sarlis *et al.*, 1992) thus suggests the possibility that elevated GC values might play a role in arthritis-induced decline in T levels.

A well characterized model of inflammation provides a valuable experimental tool to examine possible relationships between immune activation and changes in the activity of the hypothalamic-pituitary-gonadal (HPG) axis. Because much is already known regarding immunological changes present in adjuvant-induced arthritis (AIA) [see Durie *et al.*, 1994 for references], we chose this model to determine whether a phenomenon resembling rheumatoid arthritis in humans would alter gonadotropin and sex steroid secretion in the rat, and to start exploring some of the mechanisms responsible for these changes. Specifically, we determined whether AIA decreased pituitary and/or testicular responsiveness to trophic signals; whether elevated plasma levels of corticosterone participated in the observed changes; and whether nitric oxide (NO), a gas reported to influence testicular activity (Adams *et al.*, 1994) and whose levels are increased during arthritis (Stefanovic-Racic *et al.*, 1993), played a role in MBB-induced disruption of reproductive functions.

Results

Symptoms, weight gain and activation of the HPA axis

There was no measurable difference in the onset of the symptoms, or in the number of animals developing the disease, between intact or gonadectomized rats (not shown). The first signs of symptoms were consistently observed on day 11 (Figure 1). In the experiment illustrated here, 91% of the rats injected with MBB had developed swelling of the joints by day 15. In other experiments, up to 100% of the animals developed limb swelling (results not shown). Onset of symptoms (minimum score = 1) was accompanied by a significant weight loss, as previously reported (Harbuz *et al.*, 1992). While no visible and subjective signs of discomfort were present on days 7 and 10, corticosterone levels were already elevated (Figure 1).

GnRH mRNA levels

The median preoptic area (MPOA) of intact control rats or rats sacrificed 8, 12, 15 or 20 days after MBB injection (6 rats/group) contained statistically comparable numbers of hybridization signals in GnRH neurons. Specifically, there was no observable decrease in GnRH gene expression in any of the arthritic rats (results not shown).

Influence of MBB injection on LH, FSH and T secretion

Castrated rats The ability of MBB to alter gonadotropin secretion was first investigated in gonadectomized rats to facilitate measurement of gonadotropin levels. The same group of castrated rats was bled on days 7, 10, 15, 17 and 20 after injection of the vehicle or MBB. On each of these days, two blood samples were obtained over a 60 min period, and results are presented as the average of these two values. The first significant ($P<0.05$) decrease in plasma LH and FSH levels was observed on day 10, when swelling was not yet visible (Figure 2). Arthritic rats continued to exhibit lower LH and FSH values than control animals throughout the experiment.

Intact rats Plasma samples obtained on days 7, 10 and 15 indicated that intact rats injected with MBB had significantly ($P<0.01$) lower T levels than control animals (Figure 3). We

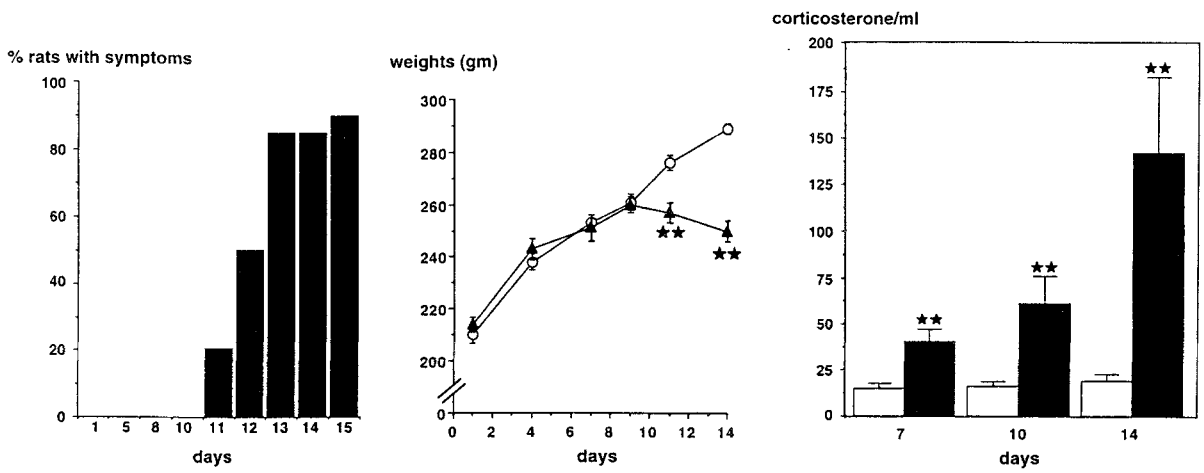

Figure 1 Development of the arthritic process: Appearance of symptoms, weight gains (○ controls, ▲ AIA rats) and plasma corticosterone levels (□, vehicle, ■ MBB). Each point or bar represents the mean ± SEM of 6–8 animals. $^{**}P<0.01$ from corresponding control

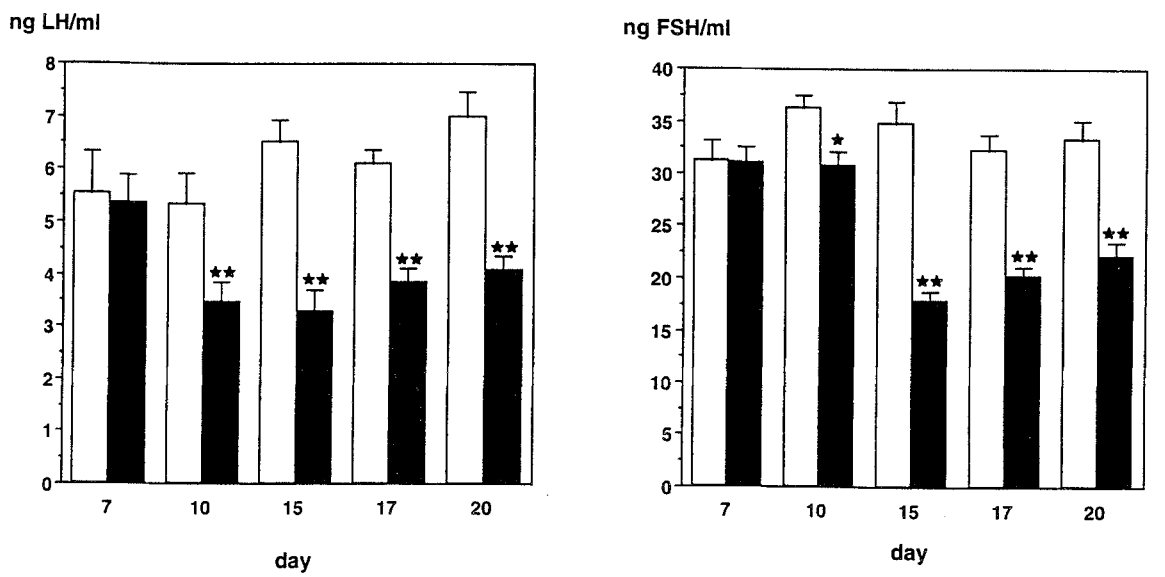

Figure 2 Plasma LH and FSH levels of castrated rats bled on days 7, 10, 15, 17 and 20 after injection of the vehicle (□) or MBB (■). Each point represents the mean ± SEM of two blood samples obtained over a 60 min period. $n = 6–8$ controls and 8–10 MBB-treated animals. $^{*}P<0.05$; $^{**}P<0.01$ from corresponding control

then determined whether there was a relationship between T levels and the absence or presence of symptoms or the severity of the symptoms. On day 15, regression analysis indicated a significant correlation between the presence and absence of symptoms ($P = 0.0093$), but no significant difference between T levels of rats with symptoms ($P = 0.191$).

Pituitary LH and FSH levels

Measurement of gonadotropin concentrations in the pituitary indicated comparable ($P > 0.05$) levels in intact control and arthritic rats on day 15 of treatment: LH, control = 6.77 ± 0.63 μg/pituitary; AIA = 7.57 ± 0.50 μg/pituitary; $P > 0.05$. FSH, control = 6.54 ± 0.65 μg/pituitary; AIA = 6.18 ± 0.33 μg/pituitary; $P > 0.05$.

Influence of arthritis on GnRH-induced LH secretion

These experiments were designed to determine whether MBB administration altered pituitary responsiveness to GnRH. In this experiment, as well as those described below, comparable results were obtained on days 9/10 or 15 of treatment. For the sake of brevity, we illustrate data collected on day 15. Both intact and castrated rats were used and yielded comparable results. Plasma LH levels were measured before, as well as 10 and 30 min after the administration of the vehicle or GnRH, and results are presented as the cumulative response over this period (Figure 4). Baseline LH levels were significantly ($P < 0.01$) lower in arthritic rats (control, 0.31 ± 0.05 ng LH/ml; AIA, 0.13 ± 0.03 ng LH/ml; $P < 0.01$). The LH response of AIA rats was decreased following administration of 200 and 400 ng GnRH/kg, but statistical significance was only reached after the former dose.

Influence of arthritis on hCG-induced T secretion

To investigate changes in gonadal function, the vehicle or hCG was injected iv to intact rats. Blood samples were obtained before as well as 45 and 90 min after acute treatment, and results are presented as the cumulative hormone response over this period. Basal T levels were significantly lower ($P < 0.01$) in arthritic than control rats (control, 2.20 ± 0.31 ng T/ml; AIA, 0.74 ± 0.09 ng T/ml; $P < 0.01$ (Figure 5). Administration of 0.2–4.0 U hCG/kg induced significantly ($P < 0.01$) higher T levels in control than in arthritic rats. In contrast, testicular responses to 4.0 U hCG were comparable between the two experimental groups.

Role of NO

Testosterone The arginine derivative L-NAME was used to probe the acute role played by endogenous NO in

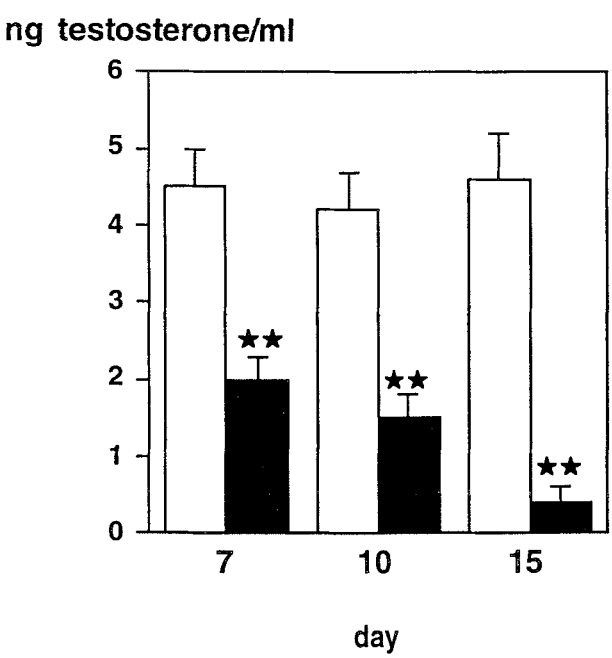

Figure 3 Plasma T levels of intact rats injected with the vehicle or MBB and bled on days 7, 10 or 15 after treatment. □, control rats; ■, MBB. Each bar represents the mean ± SEM of three blood samples obtained over a 90 min period. $n = 6$ controls and 6–8 MBB-treated animals. $^{**}P < 0.01$ from corresponding control

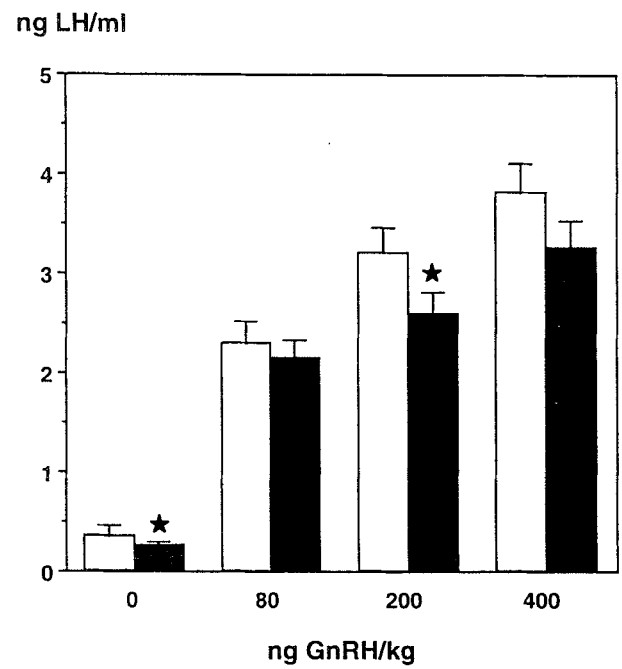

Figure 4 Effect of the iv injection of the vehicle or GnRH on LH secretion by intact control (□) or arthritic (■) rats (day 15 of treatment). Blood samples were obtained 10 min after acute iv injection. $^{*}P < 0.05$ from corresponding control

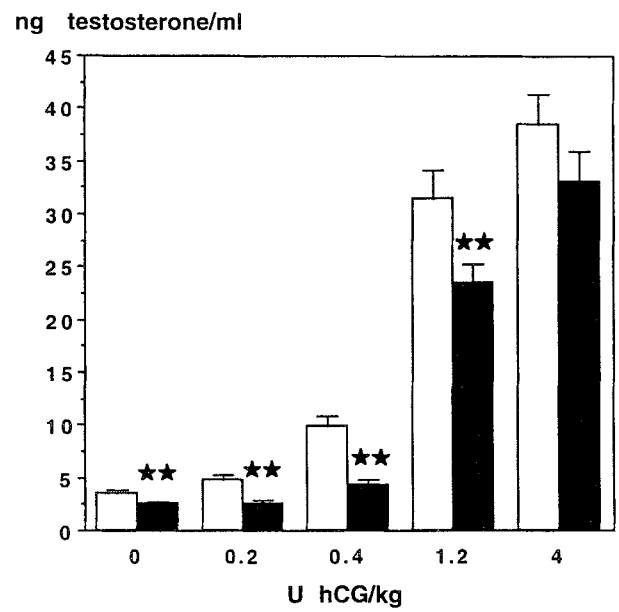

Figure 5 Effect of the iv injection of the vehicle or hCG on T secretion by intact control (□) or arthritic (■) rats (day 15 of treatment). Each point represents the mean ± SEM of 6–8 rats. $^{**}P < 0.01$ from corresponding control

modulating AIA-induced decreases in LH and T secretion. Fifteen days after MBB injection two blood samples were obtained in intact control or AIA rats. The vehicle, L-NAME (30 mg/kg), hCG (0.8 U/kg), or hCG + L-NAME were then injected, and three additional blood samples were obtained. In control rats, blockade of NO formation induced a progressive increase in baseline T levels; this increase was not present in AIA rats (Figure 6). As expected, the administration of hCG induced significantly ($P<0.01$) smaller increases in plasma T release in AIA than in control rats. Though L-NAME slightly potentiated the effect of hCG in both groups of animals, this effect did not reach statistical significance at any time.

LH Plasma LH levels of AIA rats were significantly lower than those of control animals (Control, 0.32 ± 0.04 ng/ml; AIA, 0.12 ± 0.03 ng/ml; $P<0.01$). No changes were observed in each experimental group after L-NAME or hCG administration (data not shown).

Relationship between corticosterone and T levels

Three different approaches were used to determine whether at least part of the decrease in LH and/or T levels might be related to increased corticosterone concentrations.

(a) First, we compared the temporal association between increased plasma corticosterone and decreased plasma LH/T

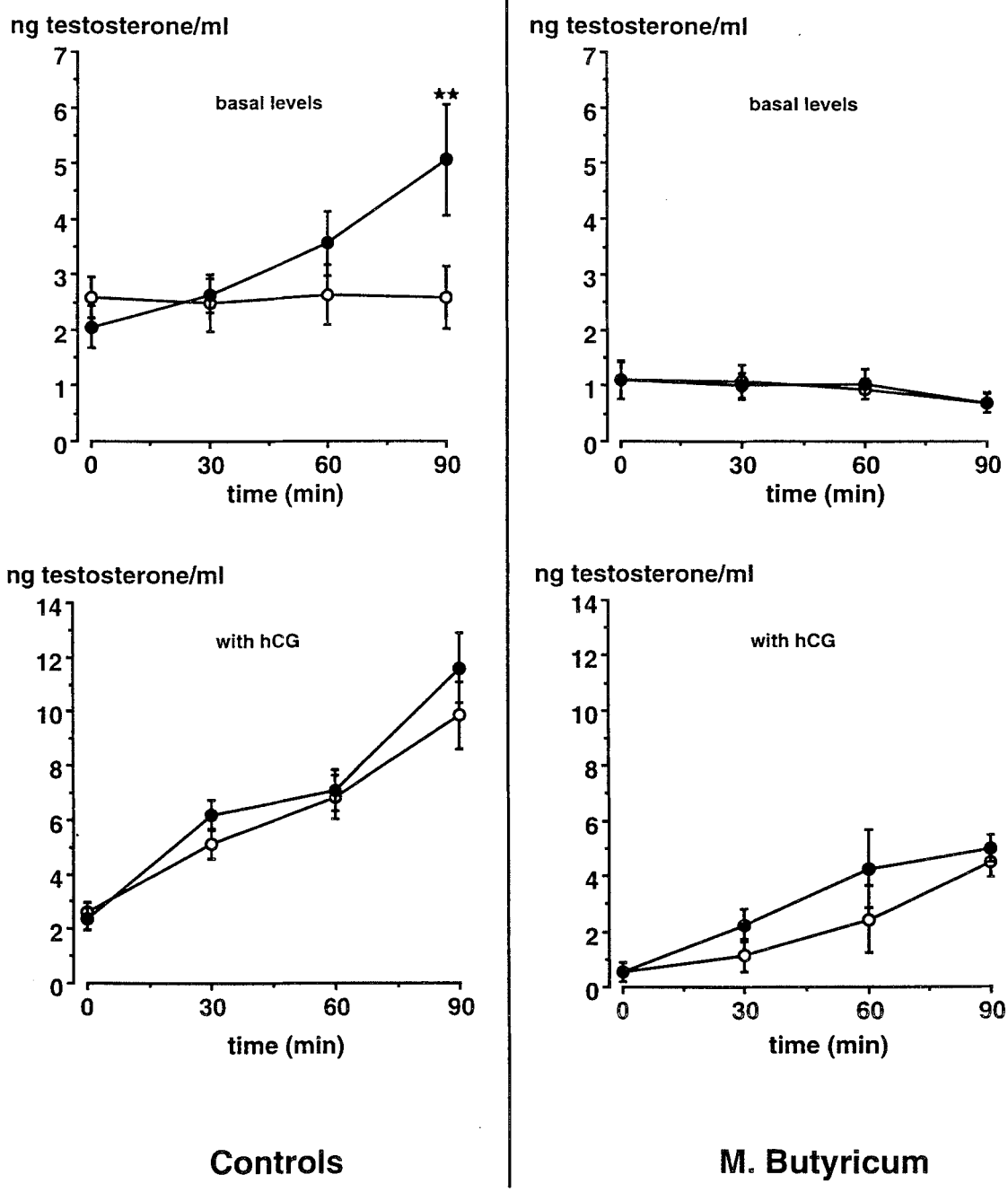

Figure 6 Effect of the vehicle (○) or L-NAME (●, 30 mg/kg) on basal and hCG-induced T secretion by intact control and arthritic rats (day 15 of treatment). HCG was administered at 0.8 U/kg. Each point represents the mean ± SEM of 6–8 animals. $^{**}P<0.01$ from corresponding control

levels. As illustrated in Figure 1, corticosterone secretion was already significantly ($P<0.05$) increased by day 7, indicating the concomitant presence of changes in the activity of both the HPA axis and reproductive functions. However, by themselves these results do not indicate a functional relationship between increased corticosterone and blunted T secretion. Two additional approaches were therefore used.

(b) In a second series of experiments, we administered CRF antibodies to AIA rats in order to determine whether decreased pituitary and/or gonadal activity would take place if significant increases in corticosterone were not present. The antibodies were injected (1.2 ml/kg, sc) on days 1, 4, 7, 10 and 13 after MBB or vehicle treatment. We have found this regimen of CRF antiserum to be fully effective in completely blocking adrenalectomized-induced rises in ACTH levels as well as corticosterone secretion induced by a brief session of mild electroshocks applied every other day over a 15-day period (C. Rivier, unpublished). In support of the concept that CRF exerts a pro-inflammatory influence (Karalis *et al.*, 1991), we observed that out of 10 rats injected with MBB and the CRF antibodies, only five developed visible swelling by day 15 of treatment. In contrast, in this particular experiment all rats administered NSS developed symptoms (8/8). In AIA rats, the acute iv injection of CRF antibodies significantly decreased plasma corticosterone levels measured 60–180 min later (ng corticosterone/ml: NSS = 91.9 ± 16.8; CRF antibodies, 17.2 ± 4.2; $P<0.01$). Chronic removal of endogenous CRF during the development of arthritis produced a small but significant ($P<0.05$) decrease in corticosterone concentrations in those animals developing the disease, though values remained clearly elevated over those of control rats (Table 1). Plasma T concentrations, which were significantly ($P<0.01$) lower in AIA rats, were not altered by removing endogenous CRF.

(c) We therefore used a third approach, in which ADX/intact or ADX/castrated rats implanted with corticosterone pellets were injected with MBB or its vehicle. On day 15 of treatment, mean corticosterone levels in ADX/intact and ADX/castrates were 13.6 ± 0.8 in control animals and 14.9 ± 0.9 in AIA rats ($P>0.05$). In ADX/castrates, LH concentrations were slightly, but significantly decreased by MBB treatment (control: 4.59 ± 0.43 ng/ml; AIA: 3.02 ± 0.45 ng/ml; $P<0.05$). In ADX/intact animals, AIA-induced decreases in plasma T levels were present despite the lack of changes in corticosterone concentrations (control: 1.45 ± 0.19 ng T/ml; AIA: 0.31 ± 0.04 ng T/ml; $P<0.01$).

Table 1 Plasma corticosterone levels of rats periodically injected with CRF antibodies

Treatment	*Corticosterone (ng/ml)*[1]	*ng T/ml*[1]
Vehicle/NSS	14.9 ± 3.1	2.87 ± 0.43
Vehicle/CRF antibodies[2]	11.3 ± 3.8	3.21 ± 0.39
MBB/NSS	75.6 ± 9.4[a]	0.51 ± 0.05[a]
MBB/CRF antibodies	55.2 ± 7.4[a,b]	0.45 ± 0.06[a,b]

[1]Corticosterone and T levels were measured at 1100, 1200 and 1300 on days 15 after treatment with MBB or its vehicle. Results shown here represent the mean of these three values. [2]NSS or CRF antibodies were administered sc (1.2 ml/kg) every 4th day, starting on day 1. [a]$P<0.01$ from vehicle. [b]$P<0.05$ from MBB/NSS. Results are shown as means ± SEM. [c]$P>0.05$ from MBB/NSS.

Discussion

We show here that arthritic male rats exhibit a significant decrease in T secretion and that at least in castrated animals, there is also a measurable blunting of LH and FSH release. Blunted pituitary activity could be secondary to altered GnRH secretion, changes in GnRH receptors, defects in post-receptor mechanisms such as impaired gonadotropin synthesis, and/or an inhibitory influence of GC. Arthritis is accompanied by marked increases in circulating cytokine levels (Matsukawa *et al.*, 1993; Sugita *et al.*, 1993; North *et al.*, 1994). As this phenomenon can lead to increased synthesis of immune signals in the brain (Ban *et al.*, 1992; VanDam *et al.*, 1992; Gatti & Bartfai, 1993; Roth *et al.*, 1993; Quan *et al.*, 1994), the well known inhibitory influence of these signals on GnRH-dependent pathways [refs. in Rivier & Rivest, 1991] might provide a mechanism through which the arthritic process decreased plasma LH levels. However, we did not observe significant changes in GnRH mRNA levels. On the other hand, the lack of increases in plasma LH levels despite very low T concentrations, indicates an inability of the gonadotrophs to respond to altered steroid feedback. This phenomenon is generally attributed to defects of the functional link between the hypothalamus and the pituitary, possibly because of increased opioid tone (Kalra & Kalra, 1983). It is therefore feasible that GnRH pulse frequency and/or amplitude may have been decreased by MBB. Long-term exposure to increases in circulating cytokines may also have blunted Gn synthesis. The fairly normal response of the gonadotrophs to GnRH, as well as the comparable concentrations of LH in the pituitary of control and AIA rats, do not, however, support this concept. Finally, elevated plasma levels of corticosterone, a condition that interferes with LH release (Ringstrom & Schwartz, 1984; Mann *et al.*, 1987), represent possible mechanisms that are discussed below.

A primary defect in the reproductive parameters of MBB-injected rats appears to be at the level of the gonads, with a dramatic decrease in both basal and hCG-stimulated T release. Our results, which are in agreement with previous reports (Bruot & Clemens, 1987, 1989), suggest that one reason for blunted androgen secretion was an impaired testicular responsiveness to LH, and that decreased gonadotropin concentrations played only an accessory role. This hypothesis was also proposed by investigators who observed that AIA rats showed low T concentrations in the presence of slightly increased LH values (Bruot & Clemens, 1987). In searching for mechanisms that could mediate the decreased testicular activity we observed, we first considered a potential role of increased GC levels. These steroids are known to inhibit Leydig cell function (Bambino & Hsueh, 1981) and are often considered important for the decrease in sex steroid levels observed during prolonged stress, possibly through disruption of specific steps of the steroidogenic pathways (Orr & Mann, 1992; Orr *et al.*, 1994). Our initial attempt to use CRF antibodies to prevent MBB-induced corticosterone release was not successful. Indeed while we could readily produce short-lived decreases in corticosterone values by the acute administration of these reagents, long-term removal of this peptide was surprisingly ineffective in this regard. This finding, which may be due to the ability of vasopressin (VP) to maintain elevated ACTH levels in inflammatory paradigms accompanied by decreased CRF synthesis in the hypothalamus (Harbuz *et al.*, 1992; Chowdrey *et al.*, 1994), prevented us from using this model to investigate the influence of adrenal steroids on the HPG axis of AIA rats. It may therefore be necessary to remove endogenous VP over the 12–15-day course of our experiments in order to determine whether this procedure restores normal T concentrations. We thus turned to ADX/corticosterone-replaced rats to determine whether clamping adrenal steroid release would prevent MBB-induced decreases in androgen secretion; and whether pituitary activity would be altered either independently from, or in conjunction with, changes in T release. T concentrations of control ADX animals with intact testes were slightly lower than those of intact rats, a finding also reported by others (Mann *et al.*, 1987). Upon injection with MBB, ADX rats showed significant decreases in T concentration despite the absence of measurable changes in the corticosterone milieu. Our results do not, therefore, support the hypothesis that elevated GC levels are important of the decreased testicular activity of rats administered MBB. Finally, we show here that clamping corticosterone levels in

AIA rats may have partially restored a normal rate of LH secretion. Consequently the known inhibitory influence of GC on LH secretion (Kamel & Kubajak, 1987; Suter *et al.*, 1988; Belhadj *et al.*, 1989) may play a role in our inflammatory model.

Arthritic rats have elevated plasma IL-6 concentrations (Holt *et al.*, 1992; Sugita *et al.*, 1993). At present, the role of endogenous cytokines in the AIA model remains inferential, but nevertheless deserves mention. We have reported that circulating cytokines act directly on the gonads to blunt sex steroid secretion (Rivier & Vale, 1989; Rivier & Rivest, 1993), a phenomenon that may be mediated by changes in the availability of cholesterol substrate and some rate-limiting enzyme (Calkins *et al.*, 1990; Lin *et al.*, 1991; Mauduit *et al.*, 1991). Whether this phenomenon participates in the decreased T release we measured will need to be explored with specific antibodies or receptor antagonists. Alternatively, increases in gonadal IL-1β levels may be important, for example by triggering testicular synthesis of CRF (Tortorella *et al.*, 1993) and thus inhibiting T production (Fabbri *et al.*, 1990). In this regard, it was of interest to note that decreased T, and to a lesser extend of gonadotrophin levels, preceded the overt appearance of limb swelling. There is little doubt that local immunological reactions start within hours of MBB injection. It is therefore feasible that early increases in circulating cytokines concentrations may be responsible for both the elevated ACTH and corticosterone values, and the inhibition of the HPG axis activity. We also noted that neuroendocrine changes appeared before any visible sign of pain or discomfort. While we did not quantitatively evaluate the level of pain, subjective indexes such as vocalization and hindered ambulation were not present when low T levels were first observed. Decreased body weight is also a fairly good index of pain and discomfort in rats, and the fact that the AIA rats showed changes in hormone levels while still gaining weight provides supportive evidence for an initial temporal dissociation between 'stress' and the suppression of the HPG axis activity. A final mechanism we explored was that played by endogenous NO, a gas reported to tonically suppress T secretion (Adams *et al.*, 1994). Blockade of NO synthase by the administration of the arginine derivative L-NAME increased basal T levels in control rats. This effect was not observed in AIA animals, and reversal of the inhibitory influence of the arthritic process on testicular function was not found. These results do not support a role of NO in the influence of the arthritic process on gonadal function. It should also be noted that in agreement with the work cited above, we failed to observe a significant influence of NO on basal LH release of either control or AIA animals.

In conclusion, we have shown that MBB injection is accompanied by low gonadotrophin and sex steroid levels in male rats, and that the changes in pituitary and testicular activity do not appear to be functionally related. In particular, LH release of testes-intact rats did not increase as the result of decreased T concentrations, which suggests a central defect in the normal feedback mechanism that regulates the activity of the gonadotrophs. Although CRF does not participate in the influence exerted by acutely exogenously administered cytokines on GnRH and LH release (Rivest & Rivier, 1993), it remains possible that this peptide may at least partially mediate the inhibitory effects of prolonged inflammatory processes on GnRH neuronal activity. Blunted testicular responsiveness to gonadotropin represents an essential feature of our inflammatory model, and is not secondary to elevated corticosteroid concentrations. While compelling evidence is still lacking regarding the influence of cytokines in our paradigm, it appears reasonable to propose that these proteins are relevant for the impaired reproductive function of AIA rat. A final comment regards the extent to which the AIA model can be regarded as a model of 'stress'. The inhibitory influence of many stressful stimuli on the HPG axis is well recognized, but it is important to note that inflammatory processes represent a separate category of homeostatic threats that have been relatively poorly studied. The general cross-talk between the immune system and neuroendocrine axes has received much recent attention, though there is a paucity of information regarding the influence of true immune activation on the activity of the HPG axis. It is probable that each 'stress' suppresses the activity of the HPG axis through its own set of mechanisms. The AIA model provides a tool with which to test hypotheses related to the role of specific secretagogues during inflammation.

Materials and methods

Animals

Sixty-five to 70-day-old male Sprague-Dawley rats were kept under standard feeding and lighting regimens (12 h light: 12 h darkness, lights on at 0630). Gonadectomy was performed under halothane anesthesia 5–6 days prior to treatment. Animals were observed daily for the appearance of symptoms, which in our colony takes mostly place on day 11, but is sometimes delayed until day 15 after injection of MBB. After symptoms of the disease developed, the food was placed on the bottom of the cage to facilitate access, and the water bottles were equipped with long spouts to permit drinking without stretching. Water consumption was monitored daily to ascertain the animals' access to liquids. Indwelling jugular catheters were implanted 48 h before blood sampling as previously described (Rivest *et al.*, 1993). All protocols were approved by the Salk Institute Animal Care and Use Committee.

All experiments shown here were repeated at least three times, and the reproducibility of the results corresponded to accepted criteria for *in vivo* experiments.

Arthritis

Arthritis was induced by an intradermal injection of 100 μl of ground heat-killed MBB suspended in paraffin oil (10 mg/ml) into the tail base. Preliminary studies indicated that administration of a lower dose decreased the percentage of animals that developed arthritis, but not the severity of the symptoms. Control rats received the oil only. The injection day was considered day 1 of treatment.

Precise clinical assessment of the disease can be done with a phlethysmometer (Sarlis *et al.*, 1992) or by limb thickness measurement by computer-assisted readout (Neidhart and Flückiger, 1992). However, the degree of swelling in AIA rats is such that observations with the naked eye can readily be made (Sugita *et al.*, 1993; Ceriani *et al.*, 1994; Durie *et al.*, 1994). We also failed to observe any relationship between the magnitude of neuroendocrine changes and the severity of the disease.

For results obtained before the appearance of the symptoms (days 7 and 10), animals were chosen randomly and all animals were incorporated in the analysis of the results. As in six different experiments, 85–98% of the animals injected with MBB develop swelling, a maximum of 15% of the rats used before day 12 would probably not have developed overt signs of arthritis. Finally, it is important to note that while we did not rigorously establish the level of pain caused by AIA, rats handled during the first 10 days after MBB injection failed to show any sign of distress, while animals with symptoms sometimes vocalized when their limbs were touched.

Adrenalectomy/corticosterone replacement

Adrenalectomy (ADX) was done under a lumbar approach while rats were anesthetized with halothane. Corticosterone pellets (25 mg/pellet; IRA, Toledo, OH) were implanted sc at the time of surgery. This supplement was necessary because

removal of corticosteroids exacerbate arthritic symptoms (Jessop *et al.*, 1994). Control rats received cholesterol pellets. Three days after ADX, MBB was injected as described above.

Reagents

MBB was purchased from VWR (Los Angeles, CA) and paraffin oil (spectrograde) from Fluka (Buchs, Switzerland). L-NAME (a non-selective inhibitor of NO synthase) and hCG were obtained from Sigma Corp. (St. Louis, MO) and diluted in apyrogenic saline. GnRH, a gift from Dr J. Rivier (The Salk Institute, La Jolla, CA), was diluted in 0.04 M phosphate buffer containing 0.1% BSA and 0.01 ascorbic acid. CRF antibodies were a gift from Dr. W. Vale (The Salk Institute, La Jolla, CA) and have been described in detail (Vale *et al.*, 1983).

Pituitary LH and FSH levels

For RIA measurement, pituitaries were removed following rapid decapitation, placed individually in 0.5 ml 0.04 M phosphate buffer (pH 7.4) and frozen in liquid nitrogen. Shortly before being assayed, the samples were thawed, sonicated, centrifuged, and the supernatants were diluted in the RIA buffer. Early recovery studies performed with labelled gonadotropins indicated consistent values of 83 ± 3%.

GnRH mRNA levels

Brains were obtained following perfusion of the rats with 4% paraformaldehyde. The GnRH cRNA probe, details of the *in situ* hybridization procedure, and the analysis of the hybridization signals, have been reported earlier (Rivest *et al.*, 1993). Thirty-μm sections hybridized with the GnRH cRNA probe (a gift from Dr. P. Adelman, Oregon Health Sciences University, Portland, OR) were used for semiquantitative analysis of hybridization signals for GnRH mRNA in the MPOA of control rats and rats injected with MBB 8–20 days earlier. Six animals were used in each group.

RIAs

All hormones were measured in duplicate unextracted plasma. LH and FSH concentrations were measured with RIA reagents provided by the National Pituitary and Hormone Distribution Program of the NIDDK (Rivest *et al.*, 1993). Plasma T values were measured with an RIA kit purchased from Diagnostic Products (Los Angeles, CA). Corticosterone levels were measured by RIA (Rivier, 1993). In our laboratory, intra- and inter-coefficients of variations are, respectively: LH, 3.0% and 5.8%; FSH, 2.2% and 3.7%; T, 2.1% and 4.0%. The T antibodies show the following cross-reactivities: 5α-dihydrotestosterone, 3.4%; 4-estren-17-ol-3-one, 20%; 11-ketotestosterone. 16%; 11β-hydroxytestosterone, 1.2%.

Statistical analysis

Hormone levels were analysed by one- or two-way analysis of variance. Duncan's multiple range test was used to assess differences between means.

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