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10. The doses of *p*-bromophenacyl bromide and mepacrine examined were 1 to 20 μ*M* and 0.1 to 5 m*M*, respectively; the doses at which 50 percent of the axons tested failed to reseal were 2 to 5 μ*M* in the former inhibitor and 1 m*M* in the latter.
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The Thymus-Adrenal Connection: Thymosin Has Corticotropin-Releasing Activity in Primates

Abstract. *Endotoxin-free thymosin fraction 5 elevated corticotropin, β-endorphin, and cortisol in a dose- and time-dependent fashion when administered intravenously to prepubertal cynomolgus monkeys. Two synthetic component peptides of thymosin fraction 5 had no acute effects on pituitary function, suggesting that some other peptides in thymosin fraction 5 were responsible for its corticotropin-releasing activity. In agreement with these observations, total thymectomy of juvenile macaques was associated with decreases in plasma cortisol, corticotropin, and β-endorphin. These findings indicate that the prepubertal primate thymus contains corticotropin-releasing activity that may contribute to a physiological immunoregulatory circuit between the developing immunological and pituitary-adrenal systems.*

Glucocorticoids have immunomodulatory effects. High concentrations of corticosteroids induce thymic involution, reduce mitotic activity in thymus-dependent (T) lymphocytes, and inhibit phagocytic activity of human leukocytes (*1*). In contrast, lower concentrations of glucocorticoids enhance thymocyte differentiation, stimulate antibody formation in vitro, and seem necessary for modulating the immune response (*2*). The regulatory mechanisms controlling this interaction between the hypophyseal-adrenal axis and the immune system are unknown. Recent studies indicate that at least two families of biologically active products of immunogenic tissues influence adrenal steroidogenesis. First, thymosin fraction 5 (TSN-F5), a family of peptides known to induce maturation of T lymphocytes and other thymic extracts, stimulates adrenal cortical secretion in rats and rabbits (*3*). Second, lymphokines appear also to elevate serum corticosteroid concentrations, while a macrophage product has been reported to be inhibitory (*4*).

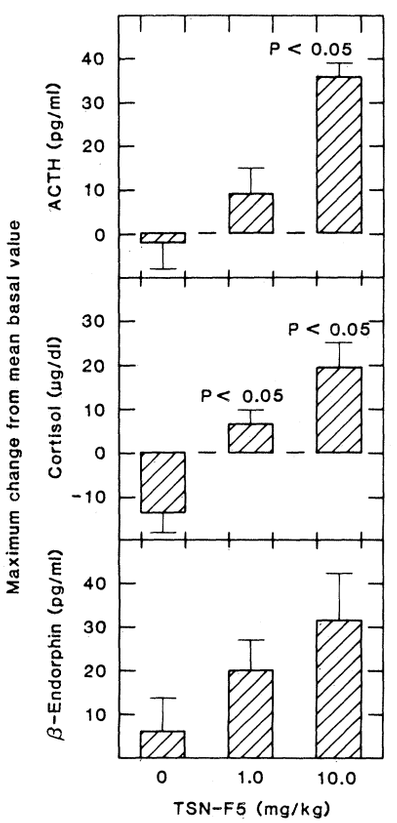

Fig. 1. Maximum change from the mean basal concentrations of plasma ACTH, cortisol, and β-endorphin after intravenous administration of TSN-F5 (1.0 or 10.0 mg/kg) or bovine serum albumin (10.0 mg/kg) in normal saline to three groups of five monkeys. Error bars indicate ± 1 standard error.

We report experiments in juvenile cynomolgus monkeys demonstrating that the thymus contains corticotropin-releasing activity. This activity may function as a subsidiary prepubertal and preadrenarcheal stimulus of the release of pituitary adrenocorticotropic hormone (ACTH). Our findings indicate a thymo-pituitary-adrenal axis in juvenile primates.

Eighteen premenarcheal cynomolgus monkeys (*Macaca fascicularis*) with a median age of 22 months (range, 11 to 27 months) were fitted with a vest and mobile tether assembly that permitted long-term cannulation of the femoral vein for serial blood collection. This allowed plasma to be harvested from monkeys that were unanesthetized, freely moving, and undisturbed by the experimenters, who were located in an adjacent room (*5*). Experiments began 24 hours after cannulation.

In the first experiment pyrogen-free TSN-F5 (10.0 or 1.0 mg/kg), crystalline bovine serum albumin (10 mg/kg), or normal saline were injected through the cannula. Plasma was collected for measurement of all anterior pituitary hormones and cortisol by radioimmunoassay (*5–7*). Red blood cells were returned to the monkeys during each experiment. Statistical significance of the changes in hormone concentrations was determined by Student's *t*-test for unpaired data. Results are expressed as means ± standard errors.

Figure 1 shows that TSN-F5 produced dose-dependent increases in plasma ACTH, cortisol, and β-endorphin; basal values were 24.06 ± 3.91 pg/ml, 35.3 ± 3.16 μg/dl, and 37.3 ± 7.65 pg/ml (N = 16), respectively. By contrast, no change in the concentration of plasma prolactin, growth hormone, thyrotropin, follicle-stimulating hormone, or luteiniz-

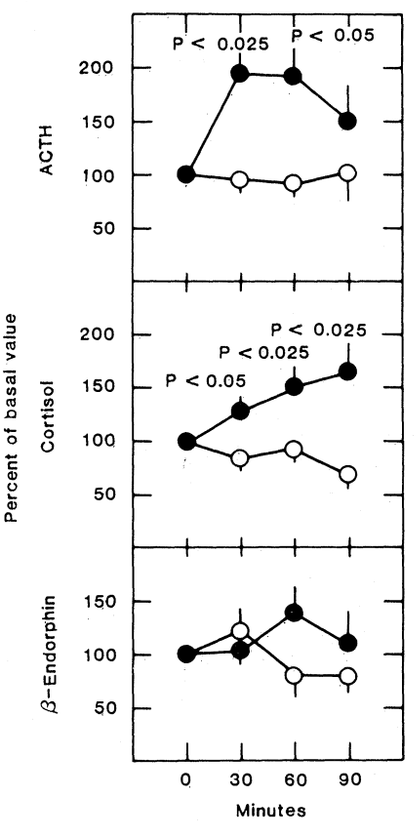

Fig. 2. Circulating concentrations of ACTH, cortisol, and β-endorphin after the administration of TSN-F5 (10 mg/kg) (●) or placebo (○) to eight premenarcheal cynomolgus monkeys at 0700 hours. Hormone concentrations are expressed as the percent of the transverse mean of 24 basal values: ACTH (22.3 ± 3.6 pg/ml), cortisol (31.9 ± 2.9 μg/dl), and β-endorphin (36.8 ± 7.6 pg/ml). Error bars indicate ± 1 standard error.

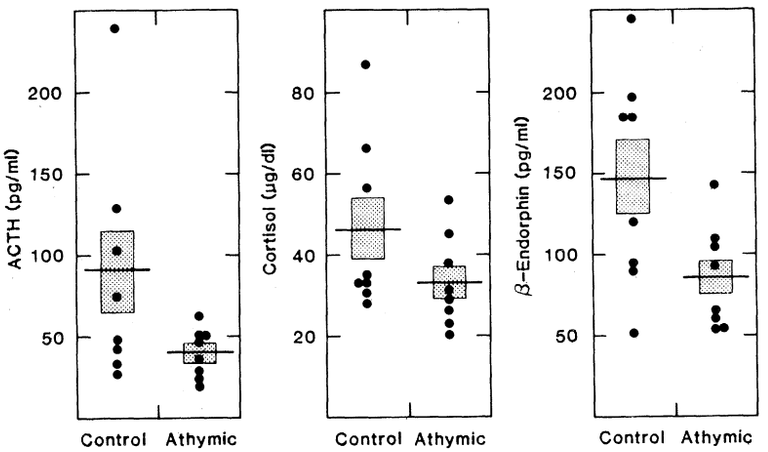

Fig. 3. Concentrations of plasma ACTH, cortisol, and β-endorphin in athymic and age-matched control monkeys. All samples were taken at 0700 hours. The shaded areas indicate ± 1 standard error. Statistical analyses are detailed in text.

ing hormone followed TSN-F5 administration.

Figure 2 depicts the time course of these responses and shows that peak levels of ACTH, β-endorphin, and cortisol occurred 30, 60, and 90 minutes, respectively, after TSN-F5 injection (10 mg/kg). These increases were similar regardless of whether experiments began at 0700 or 1400 hours. In subsequent studies, all mean hormone values had returned to the basal range 180 minutes after TSN-F5 administration.

Additional monkeys received an intravenous injection of 75 μg of thymosin-α_1 (TSN-α_1) or thymosin-β_4 (TSN-β_4) per kilogram (*3*). These two component peptides of TSN-F5 have known amino acid sequences and are suggested by murine studies to result in the release of corticosterone and luteinizing hormone, respectively (*8*). Injection of TSN-α_1 resulted in significant elevations in plasma TSN-α_1 as measured by radioimmunoassay (mean peak level, 14.7 ± 2.1 ng/ml; basal level, 1.4 ± 0.8 ng/ml; $P < 0.001$) (*9*). This increase in plasma TSN-α_1 was equivalent to that seen after TSN-F5 injection. However, in these primates TSN-α_1 administration produced no significant elevation in plasma ACTH, cortisol, or β-endorphin values and had no influence on plasma levels of other anterior pituitary hormones; TSN-β_4 was also without effect on pituitary hormone secretion. While it remains possible that a higher concentration of TSN-α_1 might stimulate the release of ACTH and β-endorphin in monkeys, these results suggest that neither of these peptides is responsible for the corticotropin-releasing activity observed with TSN-F5. Certainly there is no homology between the amino acid sequences of ovine corticotropin–releasing factor (CRF) and TSN-α_1 (*3*, *10*).

We then examined TSN-F5 for the presence of immunoreactive ACTH, β-endorphin, and CRF. Peptides were extracted from a solution of TSN-F5 in phosphate-buffered saline (1 mg/ml; *p*H 8.6) with octadecasilyl-silica cartridges, and portions with a final concentration of 250 μg/ml to 2.5 pg/ml were tested for displacement of ^{125}I-labeled peptide in radioimmunoassays for ACTH, β-endorphin, and CRF (*7*). No displacement of labeled peptides was observed.

We next chromatographed TSN-F5 (0.5 ml of a 10 mg/ml solution) on a Sephadex G-50 fine column (55 by 0.9 cm) and eluted with buffer [62 m*M* Na_2HPO_4, 13 m*M* Na_2-EDTA (*p*H 7.4), and 0.04 percent NaN_3] at a flow rate of 6 ml/hour. Fractions (0.5 ml) were collected and compared with the elution position of ACTH-(1–39) as a standard. No ACTH, β-endorphin, or CRF immunoreactivity was observed in the eluted fractions of TSN-F5 examined in this manner. In yet another experiment, monkey thymus ($N = 4$), sheep thymus ($N = 3$), and sheep pituitary were homogenized, extracted, lyophilized, and analyzed for their immunoreactive CRF, β-endorphin, and ACTH contents. No measurable CRF or β-endorphin (< 5 pg per 100 mg wet weight) was identified in thymic tissue. Small amounts of immunoreactive ACTH were detected in monkey thymus (29.7 ± 14.9 pg per 100 mg wet weight), but this was less than 0.0001 percent of the amount of immunoreactive ACTH measured in the pituitary gland.

To further evaluate the possible influence of the thymus on the hypothalamic-pituitary-adrenal axis in juvenile monkeys, we examined certain endocrine sequelae of thymectomy. We used prepubertal animals since the thymus appears functional then but atrophies after puberty. Fourteen female cynomolgus monkeys (median age, 20 months; range, 11 to 26 months) underwent total thymectomy, as verified subsequently by histological examination (*11*). The athymic monkeys were cannulated through the femoral vein at least 6 weeks after surgery. Two experiments were then performed.

First, a cohort of six primates was compared before and after thymectomy. The mean concentration of plasma cortisol (at 0800 hours) decreased from 29.0 ± 3.5 μg/dl before surgery to 19.5 ± 2.5 μg/dl afterwards ($P < 0.025$). In the second experiment eight athymic monkeys were cannulated and their hormonal profiles were compared with those of intact, age-matched control animals. Figure 3 shows that athymic primates had significantly lower levels of plasma ACTH (39.6 ± 5.7 versus 90.7 ± 26.6 pg/ml; $P < 0.05$) and β-endorphin (85.8 ± 11.3 versus 147.1 ± 24.0 pg/ml; $P < 0.025$). Although showing the same trend, plasma cortisol values were statistically equivalent (33.1 ± 4.0 versus 46.5 ± 7.6 μg/dl; $P > 0.10$) between the two groups. Plasma concentrations of other anterior pituitary hormones were also similar.

Collectively, these data indicate that the prepubertal primate thymus can stimulate secretion of ACTH, β-endorphin, and cortisol. The endocrine thymus of juvenile primates may contain a peptide that contributes to the total input stimulating ACTH secretion and adrenal function. Processing of pituitary pro-opiomelanocortin and release of its component peptides ACTH and β-endorphin might proceed directly under the aegis of such a thymic corticotropin-releasing substance (*10*, *12*). Alternatively, processing of pro-opiomelanocortin might follow thymic peptide stimulation of the release of hypothalamic CRF.

The changes in ACTH and cortisol following TSN-F5 administration did not appear to result from a nonspecific stress. No significant elevation of ACTH, β-endorphin, or cortisol followed injection of bovine serum albumin or saline. TSN-F5 is endotoxin-free and has been used in clinical trials without eliciting pyrogenic or other side effects (*3*). Furthermore, cannulation of the femoral artery in two monkeys in this study showed no change in heart rate after TSN-F5 administration.

The thymic corticotropin-releasing ac-

tivity in TSN-F5 is clearly due to a peptide, based on the biochemical preparation and detailed analysis of this fraction (*3*). As we could find no bovine CRF immunoreactivity in thymus or TSN-F5, a determination of the precise structure of this peptide awaits further purification. In addition to hypothalamic CRF, numerous other secretagogues release ACTH, including vasopressin, norepinephrine, fragments of hemoglobin, sauvagine, and urotensin I (*12, 13*). Sauvagine and urotensin I have not been isolated in mammals, while norepinephrine is unlikely to be present in TSN-F5 preparations (*3*). Clearly, in vitro studies adding subsets of TSN-F5 to corticotrophic cell cultures are now indicated to help define the structure of this peptide. It remains possible that thymic corticotropin-releasing activity resides in a vasopressin-like amino acid sequence that is either normally present in this gland or is a derivative of some larger thymic peptide. Such potentiation of ovine CRF activity by vasopressin has been demonstrated with rat pituitary cells in culture (*12*).

No ACTH activity resided in the TSN-F5 peptide fraction in our study, as tested by chromatography and radioimmunoassay, although primate thymus was shown to contain very small amounts of immunoreactive ACTH. This observation is consistent with the frequent clinical identification of a thymoma as the cause of the ectopic ACTH syndrome (*14*). However, no ACTH or β-endorphin immunoreactivity was identified in TSN-F5: the ACTH and β-endorphin contents in TSN-F5 cannot account for the elevations in plasma ACTH, β-endorphin, and cortisol that followed administration of this thymic peptide fraction.

It was recently reported that virus-infected mouse lymphocytes appear to secrete ACTH (*15*); the existence of a lymphoid-adrenal axis was suggested. Other studies indicate that rat splenic lymphocytes contain β-endorphin receptors and display both enhanced mitogenic responses as well as stimulation of natural killer lymphocyte subsets when β-endorphin is added in vitro (*16*).

Our data extend these findings into primate physiology and suggest that a thymic peptide with corticotropin-releasing activity contributes to the total CRF input impinging on the pituitary corticotroph. This peptide might well be the "tissue CRF" suggested by Guillemin *et al.* (*17*) and demonstrated by Witorsch and Brodish (*18*) in stressed rats with substantial hypothalamic lesions. A thymic or extrahypothalamic corticotropin-releasing peptide would also explain the inability to completely abolish cortisol secretion by complete transection of the pituitary stalk (*19*). An immunoregulatory circuit may therefore exist between a putative thymic CRF, pituitary ACTH, and cortisol secretion that is regulated by the thymolytic action of adrenal glucocorticoids.

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7. We extracted ACTH and β-endorphin from plasma with octadecasilyl-silica cartridges (Waters Assoc.). The peptides were eluted from the cartridges with 60 ml of acetonitrile and 0.1 percent trifluoroacetic acid (Pierce) (60:40 by volume). Samples were lyophilized and reconstituted in assay buffer (62 m*M* Na_2HPO_4 and 13 m*M* Na_2-EDTA, *p*H 7.4, containing 0.02 percent NaN_3 and 0.1 percent Triton X-100). The radioimmunoassays were performed at 4°C in assay buffer containing 250 kallikrein-inhibiting units of Trasylol per milliliter. ACTH was measured by using a specific immunoglobulin G (IgG) antibody against ACTH (IgG-ACTH-1; IgG Corp.). The detection limit of this radioimmunoassay was 5 pg/ml. The intra- and interassay coefficients of variation were 4.3 and 12.1 percent, respectively. β-Endorphin was measured with an antibody raised in rabbits against β-endorphin (antibody HS-7 at a final dilution of 1:60,000). The sensitivity of this radioimmunoassay was 10 pg/ml. Intra- and interassay coefficients of variation were 5.5 and 16.0 percent, respectively. CRF was also measured by radioimmunoassay in unextracted plasma using the same buffer system and ^{125}I-labeled synthetic ovine CRF-(1–41) as tracer. The antibody was generated in rabbits against synthetic ovine CRF (antibody HS-20 at a final dilution of 1:180,000). The detection limit was 25 pg/ml. Intra- and interassay coefficients of variation were 5.0 and 13.0 percent, respectively.
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20. These findings were presented in part at the Meeting of the Endocrine Society, San Antonio, Texas, 8 to 10 June 1983. This work was supported in part by grants or gifts from the National Cancer Institute (CA 24974), Hoffmann–La Roche Inc., and Alpha 1 Biomedicals, Inc. D.L.H. is an Applied Health Science Fellow of the National Health and Medical Research Council of Australia. H.M.S. is a postdoctoral research fellow of the Deutsche Forschungsgemeinschaft.

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