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ACTIVE IMMUNIZATION WITH STEROIDS AS AN APPROACH TO INVESTIGATING TESTICULAR AND ADRENAL FEEDBACK CONTROL

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SUMMARY

Active immunization with steroids leads to neutralization of their biological effects since the hormone in serum is rendered unobtainable to receptors by binding to circulating antibodies. Therefore, if the steroid plays a role in gonadal or adrenal feedback control, immunization must result in increased gonadotrophin or ACTH secretion, inducing gonadal or adrenal hypertrophy and hyperfunction. We exploited this phenomenon to study the importance of various steroids in gonadal or adrenal feedback control. Groups of rabbits were immunized with different steroid conjugates and the morphological and functional alterations of the testes and adrenals were recorded. We thereby confirmed the role of testosterone and corticosterone in testicular and adrenal feedback control. Estradiol is also active in testicular feedback, but appears to act only on LH and not on FSH. In addition to corticosterone, dehydroepiandrosterone and aldosterone are of importance in adrenal feedback. Immunization against dihydrotestosterone and androstenedione yielded equivocal results because of high cross reaction of the respective antisera with other androgens. In general, the specificity of the antisera is a limiting factor of this immunological approach for the study of feedback control mechanisms.

Since the early studies of Lieberman *et al.*[1] and Neri *et al.*[2] it has been known that the biological activity of steroids can be neutralized by antisera. This effect of antisera to steroids can be exploited for the study of feedback control mechanisms. The role of a given steroid in a feedback system is thereby demonstrated if its immunological neutralization results in a response of the hypothalamo-pituitary system, which in turn causes measurable effects on the peripheral gland of this system. The classical studies of feedback control use the trophic hormone suppressing effect of a certain steroid after removal of the peripheral gland and the entire steroid spectrum normally secreted by that gland as a parameter. The major difference of the immunological approach is that the animal remains intact and that the specific steroid under investigation is eliminated exclusively, provided the antibody possesses sufficiently high specificity for this steroid.

The immunological approach was first used in the study of hypothalamo-pituitary-ovarian feedback control. Thus the positive feedback effect of estrogens on the midcycle LH release could be confirmed by passive immunization with estrogens in rats [3] and hamsters [4]. By passive immunization with estrogens it could also be demonstrated in rats that the midcycle prolactin peak is induced by estrogens [5]. More recently, active immunization with estrogens has been used for the study of ovarian feedback in sub-human primates [6, 7].

Our own investigations originated from studies on the biological effects of active immunization with testosterone, whereby the well-documented role of testosterone in testicular feedback was confirmed. We then applied active immunization with other gonadal and adrenal steroids to the study of hypothalamo-pituitary-testicular and -adrenal feedback control.

All studies were performed in sexually mature, white male New Zealand rabbits. For the production of antibodies the one-time, multiple-site intradermal immunization technique was used [8, 9]. No booster injections were given following the primary immunization. Ninetyfive % of the immunized rabbits developed antibodies, whereby highest titers were usually achieved 4–8 weeks after immunization. The investigations reported here were carried out between 6 and 14 weeks after immunization.

As a first confirmation that steroids can be neutralized by antibodies we observed that male rabbits actively immunized with testosterone-3-BSA lose sexual activity. Since estrogen-immunized rabbits were not different from controls in regard to sexual activity, the immunization procedure itself may be ruled out as effecting these alterations [10].

The accessory reproductive glands of the testosterone-immunized animals showed signs of atrophy [9] and the prostate tissue showed a reduced cyclic AMP concentration (Wombacher and Nieschlag, unpublished).

Despite this obvious lack of androgens the circulating testosterone levels in the immunized rabbits were increased 20 to 100 fold. Simultaneously, however, the percentage bound testosterone in serum had increased from 90% to almost 100% and the LH and FSH levels in peripheral blood were elevated [11, 12]. The half-life of testosterone in serum had increased almost 3 fold compared to normal rabbits (Nieschlag and Becher, unpublished).

These functional changes were accompanied by alterations in testicular morphology. The weight of

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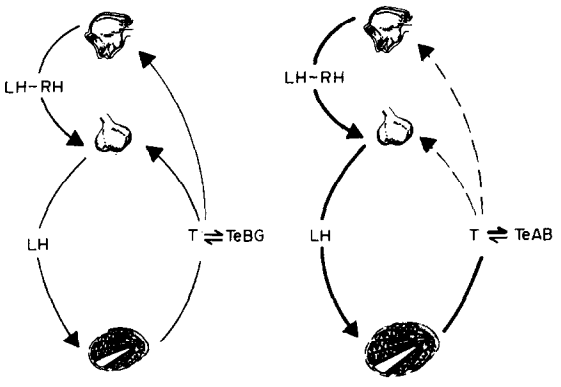

Fig. 1. Effect of circulating testosterone antibodies (TeAB) on hypothalamo-pituitary feedback. Left diagram shows situation in normal, right diagram in immunized animals.

the testes of the immunized animals had significantly increased and the increase in the percentage of Leydig cells was proportionately even greater. The nuclear volume of the Leydig cells had also increased as a sign of increased cell activity [11].

The testicular testosterone concentration and the testicular response to HCG *in vitro* had increased in the same order of magnitude as the mass of interstitial tissue [13]. Thus the endocrine capacity of the testes was augmented parallel to the increase in Leydig cell tissue. No impairment of spermatogenesis was observed, indicating that testosterone acts on the tubules before it enters the blood stream and is bound to antibodies.

These findings in animals immunized with testosterone are summarized in Table 1 and they find a common explanation by a decrease of the biologically active, free testosterone fraction in serum, which is caused by binding to antibodies in peripheral circulation. Thereby the steroid is rendered inaccessible to receptors and the hypothalamo-pituitary system reacts adequately with increased LH and FSH secretion, yielding the observed testicular hypertrophy and hyperfunction. This explanation is schematically illustrated in Fig. 1.

This explanation forms the basis for our further immunological investigations of testicular and adrenal feedback control. If a steroid plays a role in the feedback system, immunization should result in increased trophic hormone secretion inducing hypertrophy and hyperfunction of the peripheral gland, as it was observed in the case of immunization with testosterone. In the following studies [14] we were specifically interested to investigate whether a differential effect of testosterone, dihydrotestosterone and estrogens in testicular feedback could be observed after immunization with these steroids. Furthermore, the role of cortisol, aldosterone and dehydroepiandrosterone in adrenal feedback were studied by this method. The results are summarized in the following and the limitations of the immunological approach are indicated.

Male rabbits were immunized with 5α-dihydrotestosterone-3-BSA, 5β-dihydrotestosterone-3-BSA, androstenedione-3-BSA, 6-keto-estradiol-6-BSA, dehydroepiandrosterone-17-BSA, cortisol-21-BSA and aldosterone-3-BSA. Following the immunization, the peripheral concentration and percentage binding of several steroids as well as the specificity of the antibodies and serum-LH and -FSH concentrations were determined. Testicular morphology was evaluated by histometry. Adrenal activity was assessed morphologically by measurement of the nuclear volume of the cells of the zona glomerulosa, fasciculata and reticularis.

As far as it was possible to measure the various steroids in serum, all animals showed up to 100 fold increased serum concentrations and drastically in-

Table 1. Effects of active immunization with testosterone and estrogens in male rabbits

	Normal animals	Testosterone immunized animals	Estrogen
Serum values			
Testosterone (ng/100 ml)	665 ± 352	20,400 ± 11,120	882 ± 566
% Bound testosterone	90·4 ± 1·5	99·5 ± 0·2	93·1 ± 2·1
Estrone (pg/ml)	10·5 ± 2·1	8·9 ± 2·1	80·7 ± 65·1
Estradiol (pg/ml)	7·5 ± 3·2	6·0 ± 3·9	23·7 ± 11·2
% Bound estradiol	81·6 ± 2·5	92·3 ± 2·8	98·4 ± 0·8
LH (ng/ml)	3·9 ± 0·5	8·0 ± 5·1	6·1*
FSH (ng/ml)	355 ± 205	925 ± 488	370*
Half life of testosterone	16·9 ± 1·5	46·2 ± 6·1	—
Testes			
Weight (g)	2·87 ± 0·22	3·88 ± 0·79	2·77 ± 0·49
% Leydig cells	2·4 ± 0·2	6·7 ± 2·0	3·7 ± 0·8
% Tubular tissue	72·8 ± 9·4	71·3 ± 7·8	73·2 ± 6·1
Nuclear volume of Leydig cells (μ^3)	140 ± 14	191 ± 24	176 ± 19
Testosterone concentr. (ng/g wet tissue)	180 ± 95	475 ± 189	—
Testosterone production after HCG *in vitro* (μg/g wet tissue)	8·14 ± 2·31	15·13 ± 1·89	—
Accessory reprod. glands (g wet tissue)	2·53 ± 0·47	1·2 ± 0·28	2·61 ± 0·43
Sexual activity	+	∅	+

* Mean value from 2 animals.

creased percentage binding of the steroid against which they had been immunized. The antisera showed the highest binding affinity for the immunogenic steroid; critical cross-reaction with other steroids is discussed below (for details: 14).

Like the testosterone-immunized rabbits, the animals immunized with 5α- and 5β-dihydrotestosterone showed increased testicular weight, Leydig cell hyperplasia and increased nuclear volume of the Leydig cells. The estradiol-immunized animals showed an increase only in Leydig cell number and nuclear volume but not in testicular weight. Since the percentage of tubular tissue remained the same in all immunized and control animals it can be assumed that the animals with increased testicular weight also had an increase in tubular tissue mass. Analysis of the data on specificity and percentage binding revealed that the antisera to testosterone, 5α- and 5β-dihydrotestosterone showed a high cross-reaction of 8 to 100% for these three steroids, so that the effects observed after immunization with one of these steroids can be due to elimination of one or all three steroids. The estradiol antiserum, however, was highly specific for estradiol and showed no cross-reaction with androgens so that the observed effects can be considered as due to neutralization of estrogens (Table 1).

Assuming the classical concept of LH action on Leydig cells and of FSH action on tubular tissue, it could be concluded from these results that estrogens and androgens have a differential effect on LH and FSH secretion, since immunization with androgens leads to an increase of LH and interstitial tissue as well as to an increase in FSH and tubular tissue, while neutralization of estrogens leads only to an increase in LH and Leydig cells. If these observations can be confirmed in a larger number of animals they would support findings from Kalra *et al.*[15], who observed that testosterone as well as estradiol suppressed LH in castrated rats, but that only testosterone suppressed FSH. In men, however, Kulin and Reiter[16] found a decrease of plasma FSH but not of LH after estrogen administration and Gay and Dever[17] found no differential effect of testosterone or estrogens on LH and FSH secretion in male rats.

Another example of the importance of the specificity of the antisera in the animals is provided by the results from the animals immunized with androstenedione and cortisol. Like the testosterone- and the dihydrotestosterone-immunized rabbits, they showed an increase in testicular weight and Leydig cells. However, the cortisol antisera showed a cross-reaction of 7·5% with testosterone and the androstenedione antisera of 2·1% with testosterone. A correspondingly high increase of the percentage bound testosterone was observed (96·5 and 98·4% respectively). Thus the effects in these animals are likely to be caused by neutralization of testosterone rather than of androstenedione or cortisol. The effects of immunological neutralization of androstenedione would have been of special interest since Skinner *et al.*[18] found evidence that androstenedione may exert a negative feedback on gonadotrophin release.

The adrenal glands of the animals immunized with testosterone, androstenedione and estradiol were not different from controls and their antisera showed no cross-reaction, no increased binding and no increased levels of corticosteroids. This indicates that testosterone, androstenedione and estradiol play no role in adrenal feedback control.

The animals immunized with cortisol, dehydroepiandrosterone and aldosterone, however, showed a significant increase of the nuclear volume of the glomerulosa cells. The nuclear volume of the fasciculata cells of the cortisol-immunized animals was also increased. In none of the animals was a change in the zona reticularis observed.

Since corticosterone is the major product of the rabbit adrenal gland [19] and since the antisera of the cortisol-immunized rabbits showed a high cross-reaction with corticosterone (48%), the effects in the cortisol-immunized animals can be due to neutralization of cortisol as well as corticosterone. Increased ACTH secretion results and causes morphologically measurable stimulation of the adrenals. These findings would then be complementary to the well-documented blocking effect of glucocorticosteroids on ACTH (for review, see: 20, 21) and confirm their role in adrenal feedback control.

The antisera from the dehydroepiandrosterone-immunized animals are specific for DHA—or at least for 3β-OH-Δ^5-steroids [22]—and show no cross-reaction with cortisol or corticosterone. Hypertrophy of the glomerulosa cells in the dehydroepiandrosterone-immunized rabbits would therefore account for a negative effect of dehydroepiandrosterone on ACTH secretion. Since the aldosterone antisera also show only very low cross-reaction to cortisol (0·003%) and to corticosterone (0·014%), the increased ACTH secretion reflected in adrenal stimulation of the aldosterone-immunized animals appears to be caused by immunological neutralization of aldosterone (binding in serum from normal rabbits = 27% and from aldosterone-immunized = 95·3%) and confirms the role of aldosterone in adrenal feedback control.

In conclusion, it can be stated that the validity of the immunological approach for the study of gonadal and adrenal feedback control depends essentially on the specificity of the antibodies produced by the immunized animals. As the results from the animals immunized with the 6-keto-estradiol-conjugate producing highly specific antibodies to estradiol demonstrate, the method can be improved by the use of steroid conjugates yielding more specific antisera. However, the present results have demonstrated the basic principle of this approach. Finally, it should be pointed out that this approach is not limited to the study of gonadal and adrenal feedback control but may also be applied to other endocrine feedback control systems such as the pituitary-thyroidal axis as experiments with active immunization against thyroid hormones have shown [23].

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