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EFFECTS OF ACTIVE IMMUNISATION AGAINST STEROIDS UPON CIRCULATING HORMONE CONCENTRATIONS

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SUMMARY

Male and female Sprague–Dawley rats were actively immunised against testosterone-3-(O-carboxymethyl)oximino-bovine serum albumin (T-3-BSA) or oestradiol(17β)-6-(O-carboxymethyl)oximino-BSA(E_2-6-BSA). Animals were test bled at monthly intervals under ether anaesthesia from the jugular vein. Serum was used for hormone analysis by radioimmunoassay throughout. The common finding was that the presence of antisteroid antibodies was accompanied by total serum steroid concentrations showing 100–1000 fold increase over control values.

In the male, immunisation against T-3-BSA was also accompanied by elevated LH and FSH, and histological examination of the reproductive tract revealed marked Leydig cell hyperplasia but a reduction in the size of the ventral prostate. The picture in the serum from female rats resembled that from the male except that LH values did not rise. Persistent vaginal cornification was observed and the ovaries had large fluid-filled cysts although no granulosa cells could be seen lining the cysts, and corpora lutea were absent. In both sexes the 'free' circulating testosterone concentration fell over the period of immunisation but neither showed any change in serum prolactin levels.

Animals immunised against E_2-6-BSA showed certain similarities with the above group in that serum antibody titres rose to the accompaniment of elevated total oestradiol(17β) concentrations but LH was raised only in the females and neither sex showed any differences in FSH or prolactin levels. In the male, testosterone concentrations were significantly above controls in the intermediate stages of immunisation but no pathological changes were observed in the reproductive tract on sacrifice. The vaginal and ovarian picture in the female was similar to that described above although the cysts were rather smaller in size. Both sexes appeared to have slightly raised concentrations of 'free' E_2.

INTRODUCTION

The array of elegant immunoassay techniques now in common use bear witness to the revolution in steroid hormone methodology which has occurred since the first definitive descriptions of the production of steroid-specific antisera [1,2]. However, despite the enormous variety of anti-steroid sera which has been produced, the observation of any effects on the endocrine status of the host animals in which such antisera have been raised has been almost totally neglected.

Lieberman, Erlanger, Beiser and Agate[3] reported that the Schering Corp. had immunised intact cockerels with a testosterone-protein conjugate but that no effect on the androgen sensitive combs had been observed. Since the period of immunisation was of short duration (four weeks) any effect may have been sought too soon. Caldwell, Scaramuzzi, Tillson and Thorneycroft[4] immunised intact hamsters against an oestradiol-BSA conjugate over a much longer period and demonstrated a marked tendency to shorter oestrous cycles. The active immunisation of intact female rats against an oestrone-BSA conjugate before challenge with 7,12-dimethylbenz(α)anthracene caused an increased incidence of mammary tumours and a shortened period of induction [5]. Subsequently, Caldwell, Tillson, Esber and Thorneycroft[6] observed that the onset of tumour growth in intact female rats which had received an oestrogen dependent mammary adenocarcinoma implant was significantly delayed following active immunization against an oestradiol-17-BSA conjugate. However, none of these studies attempted to relate the undoubted changes in circulating hormone levels to the observed biological effects.

More recently, Nieschlag, Usadel, Schwedes, Kley, Schoffling and Kruskemper[7] have shown that active immunisation of intact male rabbits against a testosterone-protein conjugate leads to an elevation of circulating testosterone levels; hyperactivity of the testicular Leydig cells in such animals was also observed. Sundaram, Tsong, Hood and Brinson[8] actively immunised female Rhesus monkeys against an oestrone-protein conjugate and reported that plasma oestrogen levels were raised while two out of the four animals immunised became anovulatory.

Our own studies were initiated [9–11] in order to define more precisely the exact nature of changes in circulating hormone levels subsequent to active immunisation of an intact host against a steroid-protein conjugate, and further to relate such changes to any pathological effects observed in the tissues of hormone production or response. The model chosen for investigation was immunisation of the intact adult

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rat against testosterone-3-(O-carboxymethyl)oximino-BSA(T-3-BSA) or oestradiol-6-(O-carboxymethyl)oximino-BSA(E_2-6-BSA). We chose the rat as our experimental animal since it was both more practicable and economical to handle the number of animals sufficient to provide statistically viable data and moreover, kits for the determination of rat serum luteinising hormone (LH), follicle stimulating hormone (FSH) and prolactin by radioimmunoassay were available from the National Institute for Arthritis, Metabolism and Digestive Diseases (N.I.A.M.D.D.), U.S.A.

METHODS

Immunisation procedures

Adult (18 weeks) male Sprague–Dawley rats and four day cyclic females were immunised against T-3-BSA [9, 11] or E_2-6-BSA [10] according to the protocol outlined in Fig. 1. The immunogen was emulsified in Freund's complete adjuvant:saline (3:1, v/v) and 2 ml emulsion (containing 100–500 μg immunogen) injected at multiple subcutaneous and intradermal sites over the back and flanks of each animal. The dose was repeated (a) weekly for four weeks and then, after an interval of six weeks, at monthly intervals or (b) at monthly intervals throughout. Control animals were immunised similarly against unconjugated BSA.

Serum samples

Whole blood (4–5 ml) was obtained by venepuncture of a jugular vein while the animal was under light ether anaesthesia. After the blood had been left to coagulate overnight at 4°C, the serum was collected and stored at −20°C. Samples were taken immediately before the first immunisation and thereafter at approximately monthly intervals; on such occasions sera were obtained at random from females regardless of oestrous cycle phase.

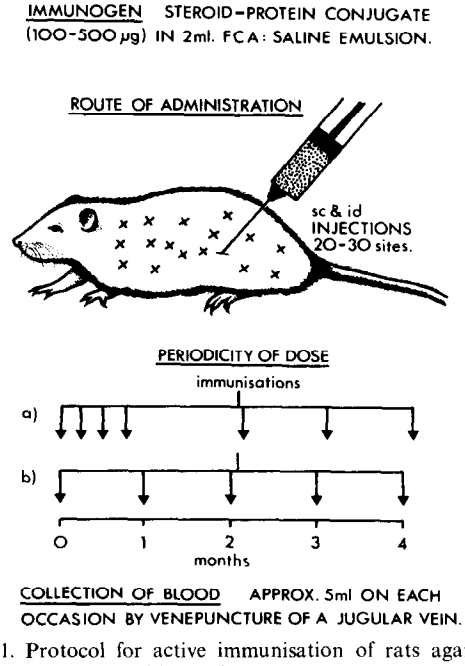

Fig. 1. Protocol for active immunisation of rats against steroid-protein conjugates.

Titration of anti-steroid antibodies in serum

Circulating anti-steroid antibody titre was expressed as that dilution of serum (final) which, when incubated overnight at 4°C with the homologous [^{3}H]-labelled steroid (30 pg [^{3}H]-testosterone or 15 pg [^{3}H]-oestradiol, bound 50% of the total radioactivity following separation of bound and free fractions with Dextran-coated charcoal [12].

Total serum testosterone concentration

The total serum testosterone concentration was determined by radioimmunoassay. Male sera were assayed using a rabbit anti-testosterone-11α-hemisuccinyl-BSA serum omitting chromatography of serum extracts [12]; sera from females were assayed with a rabbit anti-T-3-BSA serum following t.l.c. of extracts on alumina-coated sheets [13].

Total serum oestradiol concentration

The total concentration of serum oestradiol was similarly determined by radioimmunoassay [14] using a highly specific antiserum for oestradiol raised against E_2-6-BSA in a rabbit.

Serum binding capacity

The circulating protein-bound testosterone or oestradiol fraction was measured in the respective antisera employing an equilibrium dialysis technique which has been described elsewhere [9].

Serum, LH, FSH and prolactin concentrations

Immunoreactive LH, FSH and prolactin were measured in serum by double antibody radioimmunoassay using kits distributed by the Rat Pituitary Distribution Programme of the National Institute for Arthritis, Metabolism and Digestive Diseases (N.I.A.M.D.D.), N.I.H., Bethesda, Maryland, U.S.A.

Post mortem examination

At the conclusion of each period of immunisation, animals were exsanguinated and the testes or ovaries and various accessory reproductive tissues removed, weighed and fixed in Bouin's solution. Following dehydration, tissues were embedded in paraffin wax and cut into serial 5 μm sections which were stained with haematoxylin and eosin and examined under a light microscope.

RESULTS AND DISCUSSION

Active immunisation of male rats against T-3-BSA

Circulating antibodies to testosterone were detected in the experimental animals five weeks after commencing immunisation against T-3-BSA (Fig. 2a). The serum antibody titre increased after each of the three subsequent monthly injections occasionally reaching 1:28,000. Serum concentrations of testosterone (Fig.

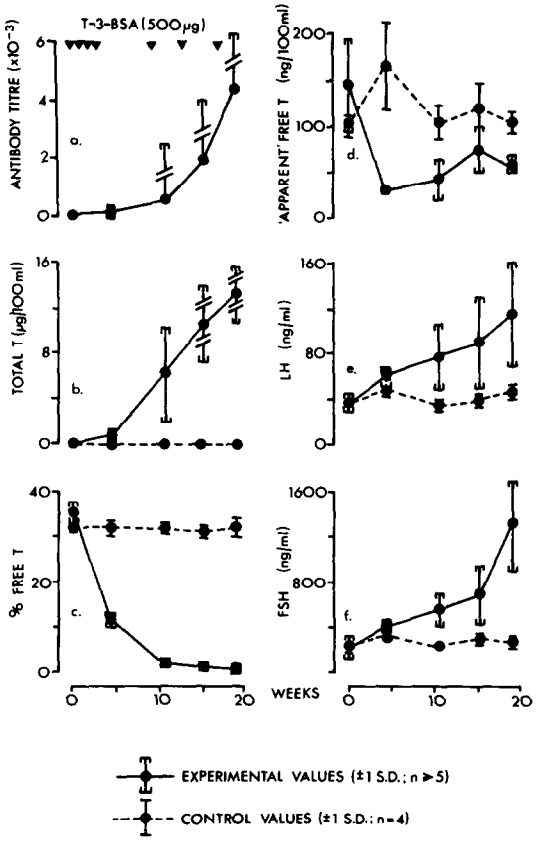

Fig. 2. Effect of active immunisation of male rats against T-3-BSA on anti-testosterone titre and serum testosterone(T), LH and FSH concentrations.

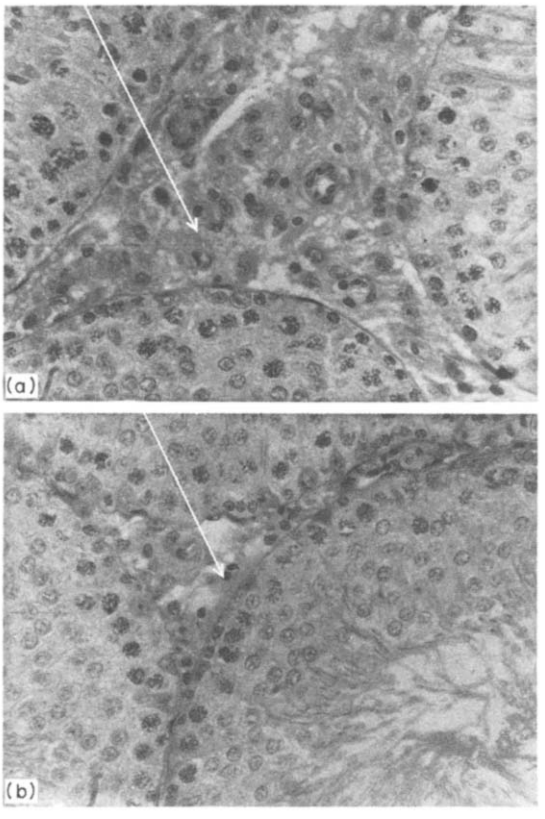

Fig. 3. Photomicrographs of testicular tissue sections from (a) rat immunised against T-3-BSA, and (b) rat immunised against BSA (5 μm sections; from × 400).
Note: the increased proliferation of Leydig cells and the enlarged interstitium (arrowed) in (a).

2b), LH (Fig. 2e) and FSH (Fig. 2f) increased over the same period of time eventually attaining grossly elevated levels compared with controls immunised against BSA, although no significant difference in serum prolactin concentrations of both groups of animals could be observed. Where sufficient serum was available the fraction of endogenous testosterone bound to circulating protein was determined by equilibrium dialysis and the results are plotted as '% free' testosterone in Fig. 2c. In the experimental group the '% free' testosterone fell steadily from 35 ± 2·8% (n = 5) before immunisation to 0·86 ± 0·4% at the final bleed when anti-testosterone titre and serum concentrations of testosterone (total), LH and FSH were maximal. Control '% free' testosterone levels remained between 33–36% throughout. The 'apparent' serum concentration of free testosterone was calculated from the '% free' testosterone and the total testosterone data and the results are plotted in Fig. 2d. It can be seen that the 'apparent' concentration of free testosterone fell dramatically over the first four weeks of immunisation from 144 ng/100 ml to 35 ng/100 ml and thereafter remained significantly lower than control values throughout. Testicular and ventral prostatic weight/body weight ratios were examined upon termination of the immunisation against

Table 1.

Effect of Active Immunisation of Male Rats against T-3-BSA on Testis and Ventral Prostate/Body Weight Ratios

Weight ratio	Immunogen: Control (NSA;N = 4)	Immunogen: Experimental (T-3-BSA;N = 10)	Significance of difference (P)
Testis/Body	5.2 ± 0.45*	6.7 ± 0.36	< 0.0005
Ventral Prostate/ Body	1.40 ± 0.22	1.04 ± 0.18	< 0.01

* Mean ± 1 Standard Deviation.

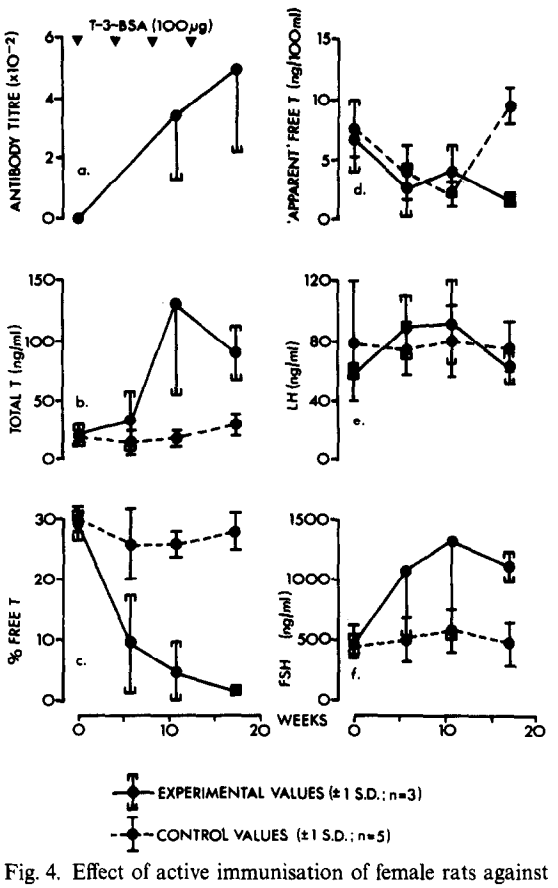

Fig. 4. Effect of active immunisation of female rats against T-3-BSA on anti-testosterone titre and serum testosterone(T), LH and FSH concentrations.

T-3-BSA (Table 1). The mean testicular weight/body weight ratio of the experimetnal group was approximately 26% greater than that of the control group whereas the ventral prostatic weight/body weight ratio was some 27% lower. Testicular histology revealed grossly enlarged interstitia with increased proliferation of Leydig cells in the T-3-BSA immunised animals (Fig. 3a); control tissues appeared normal (Fig. 3b).

These observations are in agreement with the generally accepted roles of LH and FSH as prime factors in the regulation of testicular androgen secretion. Thus, during induction of testosterone antibodies by immunisation against T-3-BSA circulating free testosterone is effectively immobilised—as evinced by the reduction in androgen-sensitive ventral prostatic weight—resulting in increased pituitary output of LH and FSH which in their turn lead to the consequent stimulation of testosterone secretion by the testicular interstitial cells.

Active immunisation of female rats against T-3-BSA

Serum anti-testosterone titre >1:20 was detected in only three of the female rats immunised against T-3-BSA; ranging between 1:350–1:700 after the seventeenth week of immunisation (Fig. 4a). The total serum testosterone concentration in these three animals throughout the period of immunisation is shown in Fig. 4b. While the total testosterone level was maximal after twelve weeks it had fallen slightly by the seventeeth week, although experimental values were still approximately four times greater than controls at this time. The '% free' testosterone fraction in sera from the same animals fell from a pre-immunisation level of 30·36 ± 1·9% to 1·94 ± 0·5% at the final bleed (Fig. 4c) at which time the 'apparent' concentration of free testosterone was significantly lower than in control sera (Fig. 4d). Serum concentrations of LH and FSH in these animals are shown in Figs. 4e and 4f; whereas FSH levels became considerably elevated neither LH nor prolactin levels differed significantly from controls. Fifteen weeks after commencing immunisation vaginal smears were taken on eight consecutive days; at this time all animals immunised against T-3-BSA exhibited persistent vaginal cornification whereas controls continued to cycle normally. Ovaries removed from the three animals which produced testosterone antibodies bore massive fluid filled cysts up to 1·5 cm in diameter. Microscopic examination of sections from these ovaries revealed large distended cysts characterised by scant or absent granulosa cells; corpora lutea were not observed (Fig. 5). Control ovaries displayed apparently normal follicular development with abundant corpora lutea.

Although the appearance of anti-testosterone antibodies leads to a demonstrable decrease in the concentration of circulating free testosterone, the response at the pituitary level was manifest by an increased output of FSH alone. This observation implies a distinct role for testosterone in the modulation of pituitary FSH output and the subsequent control of normal follicular development in the female rat.

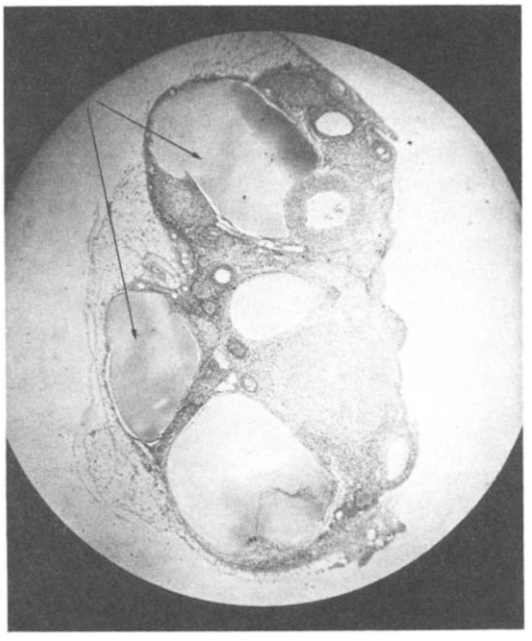

Fig. 5. Photomicrograph of ovarian section from a female rat immunised against T-3-BSA (5 μm section; from × 10). Note: the enormous follicular cysts (arrowed).

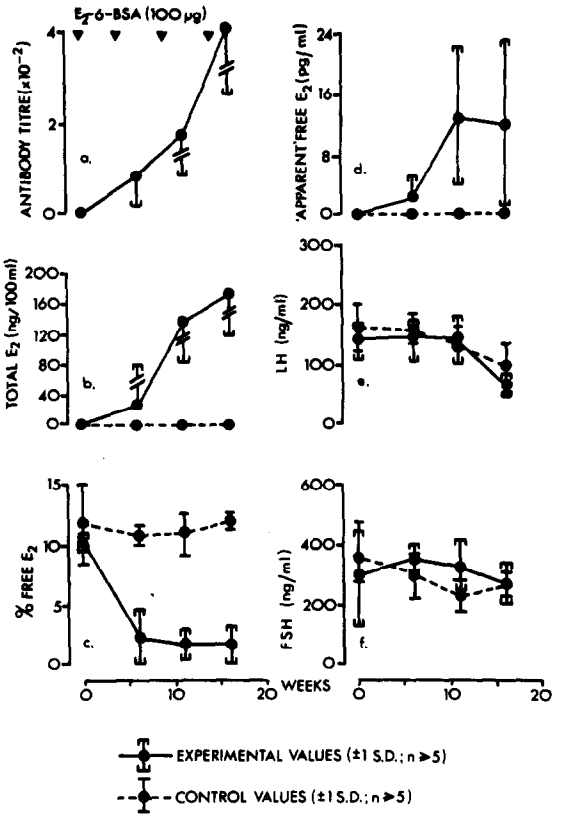

Fig. 6. Effect of active immunisation of male rats against E_2-6-BSA on anti-oestradiol titre and serum concentrations of oestradiol (E_2), LH and FSH.

Moreover, it has been suggested [15] that the polycystic ovary syndrome of women is caused by altered steroid synthesis in the ovary resulting in an imbalance of the pituitary-ovarian axis and subsequent persistent stimulation of the ovary by FSH. A detailed examination of various parameters of ovarian physiology before the appearance of polycystic ovaries in female rats immunised against T-3-BSA may therefore be of value in our understanding of the human syndrome [11].

Active immunisation of male rats against E_2-6-BSA

Following a four month period of immunisation against E_2-6-BSA the anti-oestradiol antibody titre had risen as high as 1:2200 (Fig. 6a) at which time the total serum concentration of oestradiol ranged between 7·8 and 706·0 ng/100 ml (Fig. 6b). Throughout the same period the '% free' oestradiol fraction in these sera fell from 10·13 ± 0·9% to 1·79 ± 1·39% (Fig. 6c) although this appeared to entail a nett increase in the concentration of free oestradiol (Fig. 6d). Serum concentrations of LH and FSH during this period are shown in Figs. 6e and 6f; experimental values could not be significantly distinguished from controls throughout. Similarly, prolactin levels were unaffected by the immunisation against E_2-6-BSA although testosterone levels were significantly elevated during the intermediate period of immunization (Fig. 7). However, since the serum concentration of testosterone in control animals was significantly higher than that in the experimental group before the immunisation was commenced this observation may be without value. Neither the weight nor the histology of the testes and accessory reproductive tissues removed from the E_2-6-BSA immunised animals appeared to differ from control tissues.

Any interpretation of these results is confused by the apparent increase observed in the concentration of free oestradiol following immunisation against E_2-6-BSA. However, it should be borne in mind that total serum oestradiol concentrations in the male rat are extremely low—normally between 0·2 and 0·5 ng/100 ml with 10–12% appearing as the free steroid. Following the immunisation against E_2-6-BSA they rose up to 1,000 fold, therefore a 0·05% overestimation of '% free' oestradiol in such sera could yield an estimate of free oestradiol concentration considerably higher than the total oestradiol concentration of control sera. Clearly the equilibrium dialysis technique we have used is not capable of defining the minute but critical differences in the extent of oestradiol binding displayed by such sera. Even if the immunisation was accompanied by a decrease in the concentration of free oestradiol altered pituitary gonadotrophin output would not necessarily be expected since testosterone levels remained essentially normal and would therefore have maintained the *status quo* at the pituitary level. Moreover, it seems likely that the lack of any observed biological effect following active immunisation against E_2-6-BSA was due to the presence

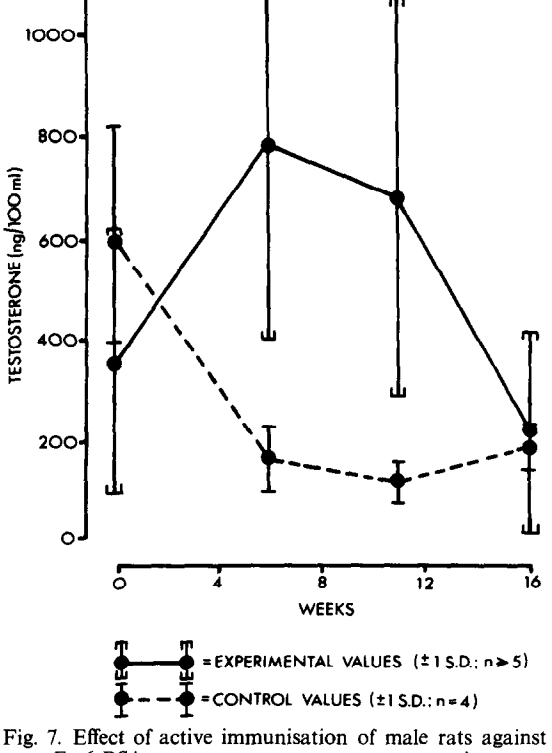

Fig. 7. Effect of active immunisation of male rats against E_2-6-BSA on serum testosterone concentration.

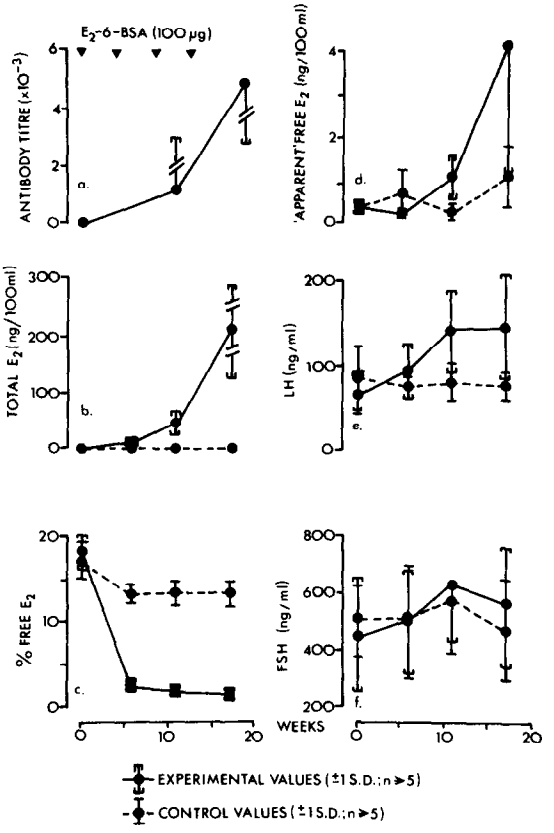

Fig. 8. Effect of active immunisation of female rats against E_2-6-BSA on anti-oestradiol titre and serum oestradiol (E_2), LH and FSH concentrations.

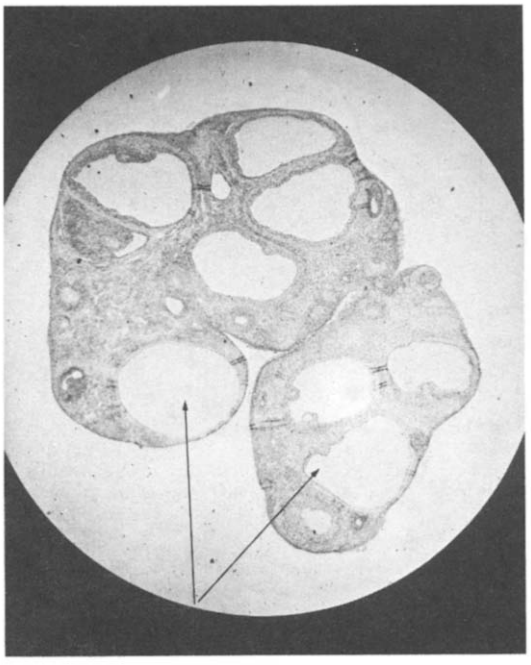

Fig. 9. Photomicrograph of ovarian section from a female rat immunised against E_2-6-BSA (5 μm section; from × 10). Note: the numerous follicular cysts (arrowed).

of a minute but adequate level of biologically active free oestradiol being maintained as a component of the equilibrium which was established between the enormous total serum oestradiol concentration and circulating anti-oestradiol antibodies.

Active immunisation of female rats against E_2-6-BSA

After the seventeenth week of immunisation against E_2-6-BSA antibody titres ranged between 1:500 and 1:20,000 (Fig. 8a) at which time total serum oestradiol concentrations had risen from 2·8 ± 3·3 ng/100 ml before immunisation to 206·7 ± 202 ng/100 ml (Fig. 8b). Once again a dramatic reduction was observed in the '% free' oestradiol fraction as the immunisation proceeded, falling from an initial level of 18·1 ± 2·4% to 1·5 ± 0·2% in the final bleed (Fig. 8c). Extrapolation of the 'apparent' free oestradiol concentration from these data suggested that the serum concentration of free oestradiol was in fact elevated in the experimental animals over the latter stages of the immunisation (Fig. 8d). However, for reasons mentioned previously it seems likely that this observation may not have been an accurate reflection of the true situation. Serum concentrations of FSH (Fig. 8f) and prolactin did not differ significantly throughout from control values although LH levels were significantly elevated nine weeks after commencing the immunisation and thereafter (Fig. 8e). Vaginal smears taken on the eight consecutive days immediately prior to sacrifice revealed that animals immunised against E_2-6-BSA had ceased to cycle and displayed constant vaginal cornification whereas controls continued to cycle normally. Ovaries removed from the experimental animals were significantly enlarged and histological examination revealed the presence of multiple follicular cysts; corpora lutea were rarely observed (Fig. 9). Uterine weight and histology did not, however, display signs of overt oestrogenic stimulation.

Sundaram *et al.*[8] reported that intact female Rhesus monkeys became anovulatory following active immunisation against an oestrone-protein conjugate. They concluded that the oestrogen dependent surge of LH necessary for the induction of ovulation did not occur because circulating antibodies to oestrone bound plasma oestrogens thereby blocking their action; plasma LH levels were not, however, quoted. The present studies shows that whereas female rats immunised against E_2-6-BSA also became acyclic, serum LH levels were in fact considerably elevated. Thus it may be that in spite of the observed binding of serum oestradiol to circulating anti-oestradiol antibodies, a level of biologically active free oestradiol, albeit minute as judged by the lack of definite signs of oestrogen stimulation in uterine tissue, remained sufficient to provide persistent stimulation of pituitary LH secretion and consequent disruption of the oestrous cycle. Moreover, chronic stimulation of the ovary by the high LH levels resulted in overt follicular cyst formation, therefore further studies with this animal model might provide yet another means for elucidating the aetiology of polycystic ovary formation in women.

In their original description of the preparation of antigenic steroid-protein conjugates Erlanger, Borek, Beiser and Lieberman[1] suggested that "antihormonal principles which could counteract the physiological effects of endogenously produced hormones would be of great importance to many phases of endocrinology", subsequently it has become apparent that the use of antibodies to steroids can indeed serve as a vital tool in the study of reproductive physiology [4, 16–22]. The present studies demonstrate that active immunisation of the intact adult rat against a steroid-protein conjugate and the observation of subsequent changes in circulating levels of that steroid and other hormones can tell us much about hormonal control mechanisms in general and related biological effects.

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