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A COMPARISON OF THE EFFECTS OF ACTIVE IMMUNIZATION OF FEMALE RHESUS MONKEYS TO ESTRADIOL-17 OR PROGESTERONE-20-PROTEIN CONJUGATES

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SUMMARY

Eleven out of 14 female monkeys were successfully immunized against estradiol-17 hemisuccinyl-BSA. The elicited antibodies were highly specific for phenolsteroids. Intervals between uterine bleedings were lengthened and anovulation occurred consistently in animals with titers above 25% binding. Circulatory estrogens were extremely elevated throughout immunization, reaching levels of 10 ng/ml or more. The disappearance rate of injected ^{3}H-estradiol was significantly prolonged in comparison to non-immunized controls.

Four out of 5 monkeys developed high titers after immunization against progesterone-20-O-carboxymethyl-oxime-BSA. These antibodies cross-reacted significantly with a number of 4-ene-3 keto steroids, e.g. 20-dihydroprogesterone, testosterone and 4-androstene dione. The length of the menstrual cycle remained unchanged in 3 out of 4 animals. More than half of the cycles remained ovulatory, as evidenced by the presence of fresh corpora lutea and increases in plasma progestins during the luteal phase.

These results indicate that estrogens are causatively related to the midcycle LH ovulatory surge, while progesterone appears not to be essential in this event. The occurrence of anovulatory cycles in monkeys immunized to progesterone-20-BSA may be explained by the lack of specificity of the antiserum.

INTRODUCTION

Over the past few years, antibodies to steroids have been demonstrated by various investigators [1–4] to be useful tools to study the role of steroid hormones in the control of various endocrine processes. In our laboratories, we have shown that such antibodies inhibited the biological effects produced by the steroid and that they did so by neutralizing the endogenous hormone in peripheral blood [5]. Our major efforts were directed towards the elucidation of the hormonal feedback mechanisms controlling the midcycle ovulatory gonadotropin surge. In rodents, passive immunization with antibodies to estradiol or progesterone was used to study the role of these hormones in this event. It was shown that in PMS-treated immature rats [6] as well as in adult cycling rats [7, 8] the proestrus ovulatory LH surge and the prolactin release were inhibited by immunization with anti-estradiol antibodies. Antibodies to progesterone did not exert such an effect on ovulation.

In this paper, an immunological approach was used to study the preovulatory hormonal feedback mechanisms in primates. The effects on the cycle of active immunization with estradiol- or progesterone-protein conjugates were compared in female rhesus monkeys. Some of the results of active immunization with the estradiol antigen were reported previously [8].

MATERIALS AND METHODS

Fourteen regularly cycling female rhesus monkeys were immunized with the estradiol antigen and 5 with the progesterone antigen. The antigens used were estradiol-17-hemisuccinyl-Bovine Serum Albumin (BSA) [8] and progesterone-20-O-carboxymethyl-oxime-BSA; they were prepared by Dr. P. E. Zimmering. 1·5–6 mg of the estradiol, and 2–3 mg of the progesterone antigens were dissolved in 0·5 ml physiological saline and mixed with an equal volume of complete Freund's adjuvant. The usual immunization schedule consisted of 4 weekly injections followed by boosters of the same doses every 6 to 8 weeks. The injections were made into 4 different subcutaneous sites near the axillary and inguinal lymph nodes.

Methods for the measurement of anti-estrogen titers, gonadotropins, plasma steroids by radioimmunoassay or protein binding assay were described previously [8]; anti-progesterone titers were determined correspondingly. The effects of various steroids on the binding of tritiated estradiol or progesterone

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by monkey antisera were determined by radioimmunoassay procedure. The percentage cross-reaction was calculated according to the method of Abraham [9].

To determine the rate of disappearance of the hormone, [2,4,6,7-^{3}H]-estradiol (New England Nuclear, S.A. 1·05 Ci/mmol) and [1,2-^{3}H]-progesterone (New England Nuclear, S.A. 33·5 Ci/mmol) were injected i.v. in controls and in animals immunized against estradiol and progesterone, respectively. Both steroids were repurified by Celite column chromatography prior to use. 2·4 2·6 μCi [^{3}H]-estradiol and 2·3 μCi [^{3}H]-progesterone dissolved in 5 ml physiological saline containing 10% ethanol were used for this experiment. Samples were taken at 0, 5, 15, 30, 60 and 90 min, as well as 2, 4, 6, 24, and 48 hours following the injection. 0·5 ml of plasma was extracted with 10 ml of ether, 9 ml of the extracts were taken to dryness and counted after addition of 5 ml of POPOP-scintillation fluid. The percentage of the injected dose per 100 ml of plasma was determined.

RESULTS

1. *Production of antibodies*

Eleven of 14 monkeys injected with estradiol-17-BSA and 4 of 5 animals injected with progesterone-20-BSA responded by producing antibodies within 2 months after the start of immunization (8, Fig. 1). Increases in titers were seen approximately 7–10 days after each booster injection.

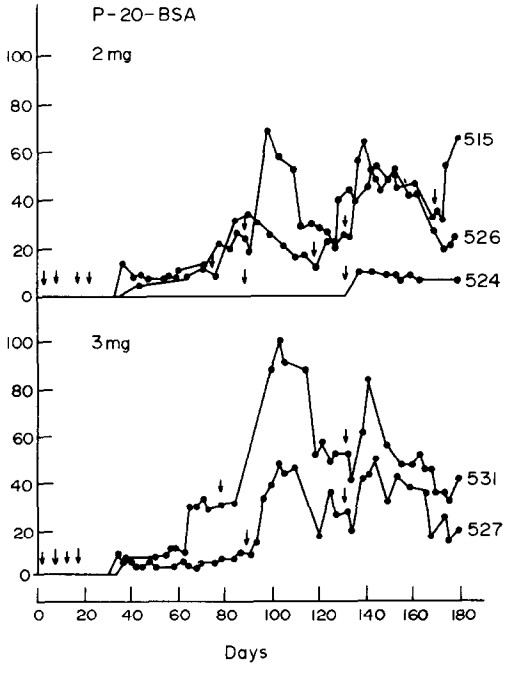

Fig. 1. Rise in anti-progesterone titers, as measured by radioimmunoassay at 1/1000 dilution, during the first 180 days immunization with 2 or 3 mg of progesterone-20-O-carboxymethyl-oxime BSA. The arrows indicate the time of antigen injection.

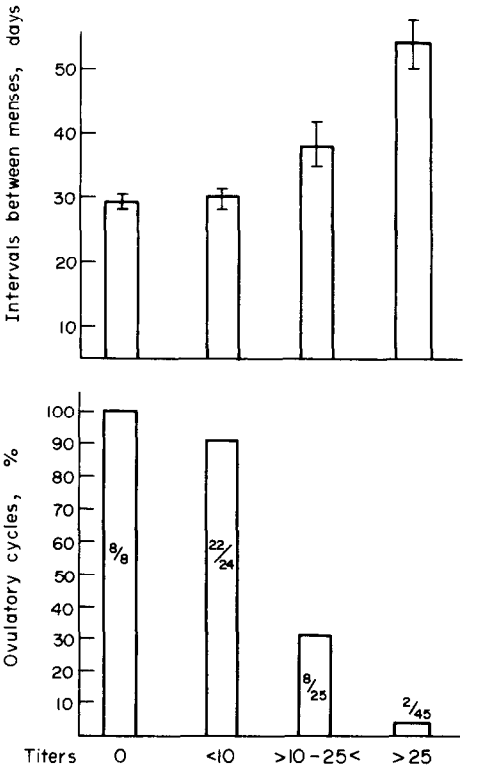

Fig. 2. Effects of immunization against estradiol on the length of ovarian cycles (mean ± S.E.) and ovulation. The bars represent the combined total of all immunized monkeys. The numerals within the bars denote the number of ovulatory cycles per total. Titers indicate % binding at 1/100 dilution as determined by radioimmunoassay.

2. *Specificity of the antisera*

Antibodies raised against estradiol-17-BSA were shown to be quite specific for phenolsteroids, with a significant amount of crossreaction against estrone and estriol, and to a lesser degree, estradiol-17α. Neither progesterone, 17-OH-progesterone, testosterone, cortisol nor diethylstilbestrol in doses up to 100 ng did compete effectively with labeled estradiol-17β for the binding sites of these antibodies [8].

Immunization with progesterone-20-BSA elicited antibodies which significantly bound 4-ene-3 keto steroids, such as progesterone (100% cross-reaction), 20α and 20β dihydroprogesterone (55% and 30%, respectively) androstenedione (12%), and testosterone (27%). 4-ene-3 keto steroids with an hydroxyl function at C 17, such as 17-OH-progesterone (5%), and cortisol (<1%), or 5-ene-compounds, such as pregnenolone (<1%) and DHEA (<1%), did not compete with [^{3}H]-progesterone for binding to the antibodies. Phenolsteroids, such as estrone and estradiol-17β, did not cross-react either (<1%).

3. *Effects of immunization on the menstrual cycle*

The mean interval between bleedings was significantly increased in all but one of the animals which developed anti-E_2 titers (Fig. 2). The cycles became

very irregular with prolonged intervals between bleeding, lasting for over 100 days in a few instances. To determine whether the immunized animals had become anovulatory, plasma progestin levels were measured 3 times a week. A cycle was considered anovulatory when progestin values remained 2 S.D. or more below the mean values seen in normal luteal phases (7·23 ± 1·64 S.D. ng/ml). Monkeys immunized to estradiol very soon become anovulatory as indicated by the absence of increases in progesterone during the last 14 days prior to menses. Anovulation in these monkeys was also confirmed by the absence of corpora lutea, as well as by the absence of cyclic changes of vaginal smears. An in-depth study of the relationship between anovulation and antibody titers, shown in Fig. 2, revealed that the percentage of anovulatory cycles is related to the titer. In order to prevent ovulation on a steady basis, the titers have to be consistently maintained above 25%. A temporary decline in titers may result in the appearance of an ovulatory cycle in between anovulatory periods. In animals with titers which were not maintained above 25% consistently, an alternance of ovulatory and anovulatory cycles can be seen. Most cycles remain ovulatory in animals with titers below 10%.

The effects of immunization to progesterone-20-BSA on the menstrual cycles are illustrated in Fig. 3. The intervals between bleedings remained unchanged, except in one monkey. In 23 out of 40 cycles, an increase in progestins was observed indicating that ovulation had occurred. Daily hormonal concentrations measured in 2 cycles revealed that the late follicular rise in estrogens was followed by an LH surge, and a rise in progestins. The presence of fresh corpora lutea was also demonstrated in histological

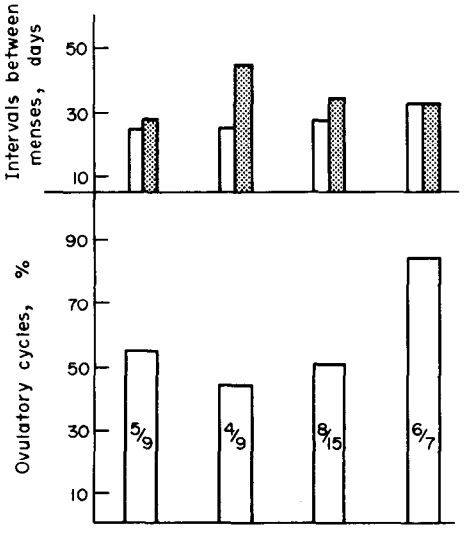

Fig. 3. Effects of immunization against progesterone on the length of ovarian cycles and ovulation in 4 individual monkeys. In the upper part, the plain and dotted bars indicate the length of the cycles before and after immunization, respectively. In the lower part, the numerals within the bars denote the number of ovulatory cycles per total number of cycles for each individual monkey.

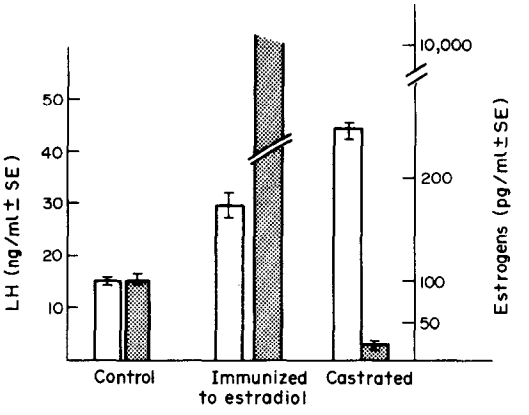

Fig. 4. Plasma LH (plain bars) and estrogen (dotted bars) levels in non-immunized controls and castrates, and monkeys immunized to estradiol. Total ether extractable estrogens were measured by radioimmunoassay.

sections of ovaries in 3 of these immunized monkeys after 1·5 years of immunization. However, in 17 out of 40 cycles, no increase in progestins was seen, thus suggesting anovulation; these anovulatory cycles occurred at various times after immunization.

4. *Steroid levels in immunized monkeys*

Three to 4 weeks after the appearance of anti-estrogen titers, the levels of estrogens increased (Fig. 4). While peak values in normal animals do not exceed 600 pg/ml during the midcycle period, estrogen levels in the immunized monkeys soon reached values of 10 ng/ml or over. High estrogen secretion was maintained throughout immunization, although some fluctuation unrelated to titers was found.

In monkeys immunized against progesterone, progestin levels remained within the normal range seen during the cycle. During the luteal phase, for instance, the levels were 6·9 (±3·9 S.D.) in the immunized animal, while the values in the control luteal phase were 7·23 (±1·64 S.D.).

5. *Half-life of the steroids*

The disappearance rates from plasma of ether-extractable [H^3]-estradiol and [H^3]-progesterone were compared in controls and in animals immunized against estradiol and progesterone, respectively. While labeled estradiol and progesterone disappeared rapidly from plasma in the controls, the immunized monkeys retained the injected radioactive steroids much longer (Fig. 5).

DISCUSSION

By immunizing female rhesus monkeys against an estradiol-17-protein conjugate, we have shown that these animals become permanently anovulatory, provided consistently high titers of antibodies are maintained. These results provide direct evidence in the primate for the essential function of estradiol in the

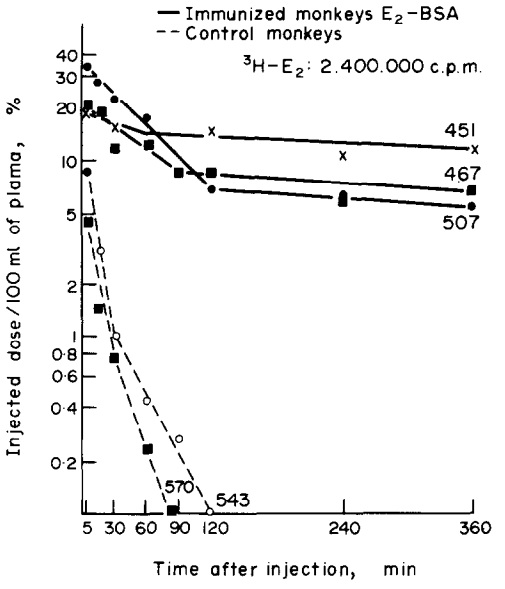

Fig. 5(A)

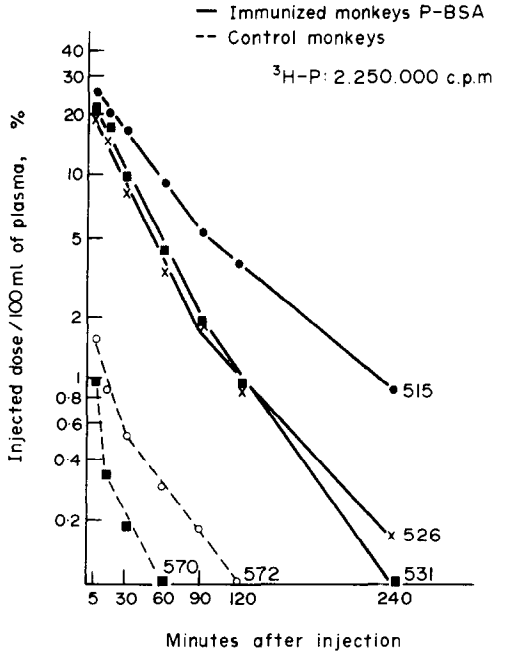

Fig. 5(B)

Fig. 5. Disappearance of extractable radioactivity from plasma of controls and of monkeys immunized against estradiol-17-BSA (A) and monkeys immunized against progesterone-20-BSA (B) after injection of [2,4,6,7-^{3}H]-estradiol-17β (A) and [1,2-^{3}H]-progesterone (B).

initiation of the LH surge, which had already been demonstrated in rodents [6, 7].

Reports in the literature regarding the physiological role of progesterone in the induction of the ovulatory LH rise are conflicting. Some investigators have indicated that administration of progesterone may lead to LH release in rats [10] and in the human [11], others have shown that progesterone derivatives may have a synergistic effect on the estrogen-induced LH increase [12, 13]. However, it has also been documented that, in rats, passive immunization against progesterone prior to the LH surge does not influence LH release or ovulation [6]. In our monkey experiments, the effects of immunization against progesterone-20-protein conjugate on the menstrual cycle are equally intriguing. 50–85% of the cycles appeared normal as far as hormone secretion is concerned. Also, fresh corpora lutea were found in these animals after 1·5 years of active immunization. These findings suggest the lack of a causal relationship between progesterone and the ovulatory LH surge in the primate. Yet, a smaller number of cycles remained anovulatory as judged by their progestin levels. Whether anovulation is a direct result of the specific neutralization of progesterone is debatable at this time. It has to be taken into account that, because of the choice of the antigen (i.e. the site of hapten linkage to the carrier protein) antibodies were elicited which cross-reacted significantly with steroids other than progesterone, such as 20-dihydroprogesterone and testosterone. The disrupting effects on the menstrual cycle seen in these monkeys may therefore be due to neutralization of steroids other than progesterone. Gay and Tomacari[14], for instance, showed that antibodies against testosterone affect FSH secretion in rats. At present, it is impossible to conclude which of the various cross-reacting and thus biologically neutralized steroids is responsible for the occurrence of anovulation. Further studies with antigens inducing the formation of more specific antibodies are required.

The high concentration of estrogens found in monkeys immunized against estradiol-17-protein conjugate most likely is the result of two different factors. First, it may reflect an increased estrogen secretion by the ovaries, which are chronically hyperstimulated by high levels of gonadotropins. The latter are obviously caused by the absence of a negative feedback effect following biological neutralization of the circulating estrogens. Secondly, the highly elevated estrogen values may be due to the prolonged half-time of disappearance found in the immunized animals, since estrogens are protected from catabolism and excretion when bound to their antibody. In contrast, progestin levels in the monkeys immunized against progesterone-20-protein conjugate remain within the normal range of the menstrual cycle. This observation may be in part explained by the fact that corpora lutea in primates have a limited secretory capacity [15, 16]. Furthermore, while active immunization increased the half-life of tritiated progesterone, this effect is definitely not as pronounced as that observed with labeled estradiol in the monkeys immunized against estrogen. One may relate this finding to the limited number of antibody-binding sites available to progesterone, which result from the competition of other crossreacting steroids.

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