Inhibition of Fetal Masculine Differentiation in the Rat by Maternal Administration of Antibodies to Bovine LH, Cyanoketone, or Antibodies to Testosterone-3-Bovine Serum Albumin[1] (37335)

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In the rabbit, it has clearly been demonstrated that the fetal testis is essential for male sexual differentiation and that fetal decapitation (hypophysectomy) impairs testicular function, leading to hypospadias. The administration of chorionic gonadotrophin can prevent this production of hypospadias (1). In the rat, the role of the pituitary during masculine development remains unclear. Raynaud (2) reported that hypophysectomy produces very slight alteration of sexual organogenesis in the rat. Furthermore, rat fetuses decapitated at the time of sexual duct development show little inhibition in masculine differentiation (3, 4). However, embryonic testes grafted in the adult exert an androgenic activity only in the presence of gonadotrophic hormone (4).

By introducing the use of highly specific "blockers" which interfere at certain steps in the developmental process we have been studying the precise mechanism of the control of sexual differentiation. In the rat, selective inhibitors of steroidogenic enzymes involved in the biosynthesis of testosterone, such as cholesterol desmolase, 3β-hydroxysteroid dehydrogenase and Δ^{5-4},3-ketosteroid isomerase, 17α-hydroxylase and C_{17-20} lyase, produce testosterone-reversible inhibition of normal masculine development (5). Antibodies, another type of specific "blocker," have been used to interfere with postnatal masculine development (6, 7). In rats, antiserum to testosterone with some cross-reactivity to dihydrotestosterone prevents testicular growth and masculine differentiation of the anogenital area *in utero* (8) and thus gives further support to the hypothesis that testosterone or one of its metabolites from the mammalian fetal testes is responsible for the transformation of the potentially bisexual fetus to a male.

It was the purpose of this investigation to study the role of the fetal pituitary–gonadal axis during the sexual development of the rat by selectively blocking interactions of the pituitary–gonadal–genitalia complex with cyanoketone (2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one), and antibodies against testosterone-3-bovine serum albumin and bovine LH.

Materials and Methods. Virgin Sprague–Dawley female rats, 8–9 weeks of age, weighing from 180 to 210 g, were mated by the supplier (Charles River Breeding Laboratories, Wilmington, MA) according to the procedure described previously (9). Six females were given an intramuscular injection of cyanoketone (60 mg/kg) dissolved in 0.1 ml of dimethylsulfoxide (DMSO) on Day 13 of gestation. An additional 12 rats were treated with antibody: 6 rats were injected, daily, on Days 13–21 of pregnancy with 0.3 ml of undiluted rabbit antiserum to bovine luteinizing hormone and 6 rats were injected with 0.1 ml of rabbit antiserum to testosterone-3-bovine serum albumin during the same period. Control females were treated with either 0.1 ml of DMSO on Day 13 of gestation or 0.3 ml of nonimmune rabbit serum on Days 13–21 of pregnancy.

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Parturition occurred naturally and the anogenital distance of male and female offspring was measured with micrometer calipers. If offspring of mothers treated with cyanoketone did not gain weight during the first two or three days of life, they were given foster mothers with the thought of improving survival. At 10 days of age, male and female rats were examined for nipples. Twenty-two days after birth the mothers were sacrificed and their organ weights were recorded. Statistical evaluation was performed by the method of Student's t. $\pm$ 1 standard deviation.

Antiserum against bovine LH was prepared in chinchilla rabbits by administration of 2.5 mg NIH-LH-B 8 dissolved in 0.5 ml normal saline and emulsified in 1.0 ml complete Freund's adjuvant (Difco, Detroit) in divided doses according to the schedule of Madhwa Raj, and Moudgal (10). The antiserum employed in this experiment was obtained ten days after the fifth injection.

Rabbits were immunized to testosterone-3-bovine serum albumin according to the modification of the method of Lieberman *et al.* (11) as previously reported (12) for an earlier study in cooperation with Doctors R. G. Wieland and J. C. Chen (8).

Results. Antibodies to testosterone-3-bovine serum albumin. The complete characterization of the antiserum has been reported (8). The testosterone binding capacity of the undiluted serum was 80 μg/ml. The reactivity of the antiserum is selectively limited to testosterone (T) with the exception of a 28% cross-reactivity to dihydrotestosterone (DHT), and, thus, is referred to as anti-T:DHT.

Antibodies to bovine LH. The antiserum does not cross-react with rat prolactin or rat growth hormone, but does bind, significantly, to rat LH and rat FSH (Table I). Therefore, the antiserum is more properly characterized as anti-LH:FSH.

Controls. No discernable differences can be found between control rats treated with DMSO or nonimmune rabbit serum, and, thus, data from these two groups are combined in the following results.

Maternal effects. The administration of either cyanoketone, anti-T:DHT or anti-LH:FSH rabbit serum to pregnant rats has no effect on body weight gain during gestation. Anti-LH:FSH treatment during Days 13–21 of pregnancy delays parturition by almost a day ($p < 0.01$) while administration of anti-T:DHT during the same period or an injection of cyanoketone on Day 13 is without effect (Table II).

At the time of weaning, 22 days postpartum, there is no significant difference in maternal body weight. However, treatment with cyanoketone results in enlarged adrenals and anti-LH:FSH causes a reduction in maternal ovarian weight (Table II).

Offspring. Regardless of treatment there is no difference in either size or number of males and females per litter. Prenatal administration of cyanoketone, anti-T:DHT or anti-LH:FSH induced nipple development and hypospadias in male offspring, but did not affect the anogenital distance of female newborns (Table III).

Discussion. The induction of hypospadias and nipple development in male rats prenatally treated with anti-LH:FSH as well as with cyanoketone and anti-T:DHT suggests that gonadotrophins, presumably of fetal

TABLE I. Characterization of Anti-Bovine LH Serum.

Rat pituitary hormone	Biological activity (IU/mg)	Anti-bovine LH serum binding capacity[a]	
		ng/ml	mIU/ml
NIAMD rat LH RP I-1	1.0	930.0	0.93
NIAMD rat FSH RP I-1	100.0	12.0	1.20
NIAMD rat prolactin RP I-1	30.0	None	None
NIAMD rat growth hormone RP I-1	1.5	6.4	0.01

[a] Determined by binding of radioiodinated purified pituitary hormone reference preparation to multiple dilutions of antiserum.

TABLE II. Maternal Effects of Cyanoketone, Anti-T:DHT and Anti-LH:FSH 22 Days Postpartum.

Group	Dose (ml)	Period of treatment (days of gestation)	No.	Length of gestation (days)	Body weight (g)	Adrenal weights		Ovarian weights	
						mg	mg %	mg	mg %
DMSO	0.1	13	3	21.2 ± 0.45[a]	303.8 ± 11.4	88.9 ± 14.5	29.2 ± 4.6	123.9 ± 20.7	40.8 ± 6.6
Rabbit serum	0.3	13–21	3						
Cyanoketone	0.1	13	4	21.0 ± 0.0	296.8 ± 16.2	111.5 ± 16.8[b]	37.5 ± 4.1[c]	147.0 ± 36.2	49.3 ± 10.6
Anti-T:DHT	0.1	13–21	6	21.0 ± 0.0	297.9 ± 17.6	80.9 ± 12.2	27.1 ± 3.6	116.4 ± 22.7	38.4 ± 6.2
Anti-LH:FSH	0.3	13–21	5	22.0 ± 0.0[c]	323.4 ± 22.7	84.5 ± 14.3	26.0 ± 2.9	96.1 ± 13.2[b]	29.8 ± 4.4[c]

[a] ± one standard deviation.
[b] $p < 0.05$.
[c] $p < 0.01$.

origin, are involved in the testicular control of masculine organogenesis.

The effects of cyanoketone on the perineum and nipples have been explained as an inhibition of fetal testicular production of Δ^4-3-keto androgens (testosterone) at the level of Δ^5-3β-hydroxysteroid dehydrogenase (5, 13). The production of hypospadias by anti-T:DHT has been attributed to an antibody-induced reduction of circulating testosterone (8). The present finding of nipple formation in males prenatally treated with anti-T:DHT extends our earlier reports (8, 13) and taken together with Neumann's observations (14) that the antiandrogen, cyproterone acetate (1,2α-methylene-6-chloro-Δ^{4-6}-pregnadiene-17α-ol-3-20-dione-17α-acetate) produces these same defects suggests that a blockade of the production, circulation or target organ binding of testosterone (or dihydrotestosterone) prevents masculine differentiation.

The administration of anti-LH to pregnant rats before Day 12 of gestation has been reported to cause vaginal bleeding and resorption of fetuses (6, 10) by reducing ovarian progesterone secretion and enhancing 20α-hydroxyprogesterone levels (6, 15). In agreement with our findings, treatment with anti-LH after Day 13 of gestation failed to interrupt normal pregnancy (6, 10) but did delay parturition (10).

In the adult male rat, neutralization of endogenous LH inhibits androgen production (16) probably by reducing circulating levels of biologically active LH. The report (7) that postnatal administration of gonadotrophin antiserum to male rats results in phallic underdevelopment agrees with our findings of hypospadias in prenatally treated male rats.

Although the control of the fetal testes by the pituitary has been shown to operate in the rabbit (1), a role for the fetal pituitary in the sexual organogenesis of the rat has not been firmly established (17, 18). The present findings clarify previous studies (2–4) by providing evidence that gonadotrophins play a role in the masculine development of the rat. A possible interpretation of our results is that the blockage in masculine development is mediated by the transplacental passage of the anti-LH:FSH which

reduced the level of circulating LH and/or FSH in the fetus which, in turn, results in a reduction of androgen output by the fetal testes below the critical level required for masculine differentiation of the perineum and inhibition of the mammary gland anlagen. The antibody is in the gamma globulin fraction which is known to pass the placenta (19).

It is possible that the gonadotrophins responsible for masculine development in the rat are fetal, maternal, or placental in origin. It is unlikely that maternal gonadotrophins pass through the placenta (18) and in the case of the rabbit in which fetal hypophysectomy results in retarded masculine organogenesis this seems to be true (1). Rat placental tissue grafted in the testes of prepubertal rats displays no gonadotrophic activity (20). Gonadotrophins have been isolated from the fetal pituitaries of numerous species (21–23) and the rat fetal testis does respond to exogenous gonadotrophins (4, 24). Thus, it seems reasonable that the pituitary–testicular axis is functioning in the rat fetus and plays a role in androgen-dependent development of target organs, such as the anogenital area and mammary glands.

Prenatal administration of cyanoketone or either antibody has no effect on the genitalia of the female offspring. Antiserum to testosterone-3-bovine serum albumin does not affect feminine differentiation (8) and although cyanoketone has been shown to induce a small increase in the anogenital distance of female fetuses (25) the total dose used was almost ten times the amount administered in this experiment.

Maternal adrenal enlargement 30 days after an injection of cyanoketone (22 days postpartum) is probably due to persistent enzyme inhibition by the retained inhibitor (26). The administration of LH antibodies reduces ovarian weight (10, 27). Following parturition, ovulation occurs in the rat and a new crop of corpora lutea are formed. The smaller ovaries of mothers treated with anti-LH:FSH 22 days before sacrifice may have been a result of an interruption in the formation or maintenance of these postpartum corpora lutea by the antibody, which had been admini-

TABLE III. Effects of Prenatal Administration of Cyanoketone, Anti-T:DHT and Anti-LH:FSH on Offspring.

Group	Dose (ml)	Period of treatment (days of gestation)	No.	Anogenital distance (mm)		No. of pregnant females	Pups	Males	Females
				Male	Female		per litter		
DMSO	0.1	13	16	4.17 ± .21[a]	1.07 ± .09	3	10.3 ± 2.3	4.8 ± 2.2	5.5 ± 1.1
Rabbit serum	0.3	13–21	16			3			
Cyanoketone	0.1	13	22	3.68 ± .29[b]	1.03 ± .12	6	8.8 ± 2.9	4.8 ± 1.7	4.0 ± 3.0
Anti-T:DHT	0.1	13–21	32	3.67 ± .23[b]	1.06 ± .10	6	10.7 ± 1.9	5.3 ± 2.2	5.3 ± 1.6
Anti-LH:FSH	0.3	13–21	27	3.85 ± .22[b]	1.09 ± .10	5	10.6 ± 2.2	4.8 ± 2.6	5.8 ± 1.6

[a] ± Standard deviation.
[b] $p < 0.001$.

stered during the 9 days just preceding delivery.

Summary. The prenatal administration of antibodies to bovine LH induces the same degree of hypospadias and nipple formation in newborn male rats as prenatal treatment with cyanoketone or antibodies to testosterone-3-bovine serum albumin, indicating a role for gonadotrophins in the testicular control of masculine organogenesis of the rat. Twenty-two days following parturition the adrenals are enlarged in mothers injected with cyanoketone during pregnancy while antibodies to bovine LH cause a reduction in maternal ovarian weight.

Bovine luteinizing hormone employed for immunization and purified rat pituitary hormone preparations utilized for characterization of anti-bovine LH serum were obtained from the Rat Hormone Distribution Program through the Hormone Distribution Officer, NIAMD, NIH.

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