Increased Secretion of Müllerian Inhibiting Substance after Immunological Blockade of Endogenous Luteinizing Hormone Releasing Hormone in the Rat*

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Summary

In order to evaluate the role of leuteinizing hormone releasing hormone (LHRH) and gonadotropins in the control of secretion of Müllerian inhibiting substance, groups of pregnant rats were injected intraperitoneally with high potency antiserum from a rabbit immunized with LHRH. Pregnant females were injected at 13 and 20 days of gestation with LHRH antiserum or an

equal volume of normal rabbit serum as a control. The testes and penis were reduced in weight and size in 6-day-old pups of the experimental group indicating the luteinizing hormone (LH) was blocked and that LHRH antiserum was active. Testicular fragments of 17-day fetuses and 6-day-old pups showed an increase relative to controls in Müllerian inhibiting substance activity in a graded organ culture bioassay system. These studies suggest that secretion of Müllerian inhibiting substance is curtailed by one or both gonadotropins.

* This research was supported in part by USPHS Grants AM-07039-02 and CA17393-02.

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Speculation

The secretion of Müllerian inhibiting substance is under inhibitory gonadotropin control, dependent on normal hypothalamic secretion of LHRH.

Two hormones govern the embryonic development of male genitalia. Müllerian inhibiting substance, first described in mammals by Jost (15,16) causes regression during mid-gestation of the Müllerian ducts of male fetal development in the rat (19,20). This hormone is secreted by the testis from day 14 of gestation until birth and in progressively lesser concentrations, until 21 days after birth (8). The second hormone, testosterone, induces development of the Wolffian duct into the vas deferens, seminal vesicle and epididymis (9); and its active metabolite, 5-dihydrotestosterone, stimulates the urogenital sinus, genital tubercle, genital folds, and genital swellings to form prostate, glans, penis and scrotum (9).

It is not certain whether Müllerian inhibiting substance is autonomous or whether it responds to extragonadal hormonal influences. Studies of the pituitary control of mammalian Müllerian inhibiting substance have been inconclusive. Maraude *et al.* have shown an increase in Müllerian inhibiting substance activity of the 2 month old chick testes after hypophysectomy at 1 month (17, 18). Groenendijk-Huijbers and Burggraaff noted a spontaneous return of Müllerian inhibiting substance activity in the testis of the 4 month old chick (10). Rats hypophysectomized at 20 days of age failed subsequently to demonstrate testicular Müllerian inhibiting substance activity (8). Neither prolactin (Donahoe *et al.*, unpublished) nor placental fragments (8) added to the *in vitro* organ culture system influenced Müllerian inhibiting substance activity.

Previously, we used luteinizing hormone releasing hormone (LHRH) antiserum to block endogenous LHRH secretion in the neonatal rat (3,4,5). Both serum concentration and pituitary content of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were reduced after injection of LHRH antiserum (2,14).

In this study, pregnant rats and their pups were treated with LHRH antiserum in order to examine whether LHRH and/or the gonadotropins influence Müllerian inhibiting substance secretion.

Methods and Materials

Animals. Adult rats were obtained from Holtzman Laboratories. Females in estrus had been caged with males overnight and those with sperm positive vaginal smears on the following day were kept for study. The animals were housed in a temperature-controlled room with a daily lighting schedule of 14 hours of light and 10 hours of darkness and had free access to Purina rat chow and tap water.

Bioassay. The presence of Müllerian inhibiting substance was assayed by a graded organ culture method (7). The agonalad reproductive ducts from 14 1/2 day female fetuses were placed on an agar coated stainless steel grid of a Falcon 3010 organ culture dish. One to 2 mm fragments of testicular tissue to be assayed were placed adjacent to the ducts. Incubations were performed over wells containing 2 ml of media (CMRL 10% fetal calf serum 200 units penicillin and 200 mcg streptomycin) (Gibco) at 37°C for 72 hours in a humidified atmosphere of 95% air and 5% CO₂. Specimens were then fixed in buffered formalin, dehydrated in an alcohol series, cleared in xylene, and embedded in paraffin. The cranial end of the duct was cut in serial cross-sections and stained with hematoxylin and eosin. Multiple sections were studied by light microscopy and regression of the duct graded on a scale of 0 to 5. The mean of two observations by independent experienced observers was recorded.

LHRH Antiserum.

Antiserum to LHRH was generated in rabbits by repeated intradermal/subcutaneous injections of synthetic LHRH conjugated to bovine thyroglobulin by bisdiazotized benzidine (Jackson, in preparation). This technique was similar to that used to generate antibodies to thyrotropin releasing hormone (12). For the initial injection, the complex was emulsified in Freund's complete adjuvant; subsequent injection utilized incomplete adjuvant. The LHRH antiserum showed no cross-reactivity with deamido-LHRH, thyrotropin releasing hormone, somatostatin, vasopressin, triiodothyronine, thyroxine, or angiotensin I or II. The antibody reacted with the C-terminal nonapeptide to pentapeptide fragments of LHRH but not significantly with N-terminal fragments. A 1:280,000 dilution of LHRH antiserum bound 45% of labelled LHRH under the conditions of our LHRH radioimmunoassay (Jackson *et al.*, in preparation).

Experiment Protocol. Effect of LHRH antiserum on fetal and neonatal male rats

Thirteen day pregnant females were chosen for injection because LHRH antiserum did not interfere with normal continuation of pregnancy (2) and Müllerian duct regression had not begun in normal male fetuses (7).

In vitro (Figure 1). Ten 13-day old pregnant rats were given 1 ml of LHRH antiserum intraperitoneally, and another ten rats were treated with an equal volume of normal rabbit serum. At 17 days gestational age, male fetuses were removed from each of 2 experimental and 2 control mothers. One testis from each male fetus was removed and a small fragment placed aseptically on an agar coated stainless steel grid adjacent to the urogenital ridge of a 14 1/2 day female rat embryo for Müllerian inhibiting substance assay. The remaining pregnant females were injected intraperitoneally with a booster of 1 ml LHRH antiserum or normal rabbit serum on the 20th day of pregnancy. After delivery the males were carefully marked as LHRH antiserum or control pups, then were randomly assigned to either experimental or control mothers in equal numbers. At six days of age, 5 experimental and 5 control animals were killed. The animals were weighed, killed and the testes removed. One testis was weighed and duplicate 1-2 mm fragments of the other evaluated for Müllerian inhibiting substance assay. The penis was also removed and measured under a Nikon dissecting microscope.

Six-day old male pups from a mother treated with LHRH antiserum were given 0.25 ml LHRH antiserum intraperitoneally, and similarly, the remaining equal number of male pups from a mother which had received normal rabbit serum were given 0.25 ml normal rabbit serum intraperitoneally. At 10 days of age, the animals were weighed, their testes and penises removed as described for 6-day old animals.

In vivo. In order to determine if early changes in Müllerian inhibition could be elicited *in vivo*, a smaller group of 6 experimental and 6 control pregnant females were injected with one ml of LHRH antiserum or normal rabbit serum on day 13 of gestation. Two experimental and two control mothers were killed on day 14, 15 and 16 of gestation and the male pups were removed. One urogenital ridge from each of the pups was removed, fixed in buffered formalin, embedded, and stained as described above. Müllerian duct regression was evaluated directly without further *in vitro* incubation.

Statistical Analysis

Group comparisons were analyzed with unpaired t tests. All data are presented as mean $\pm$ standard error (SE).

RESULTS

Müllerian inhibiting substance activity. Organ culture of testes from 17-day old male fetuses from dams treated with LHRH antiserum at 13 days fetal age had significantly increased Müllerian inhibiting substance (Grade 4.9 $\pm$ 0.1 vs. 3.8 $\pm$ 0.5, $p < 0.05$) (Figures 2 and 3). Similarly, the testes from 6-day old pups from mothers which had received LHRH antiserum at fetal ages of 13 and 20 days had increased Müllerian inhibiting substance activity (Grade 4.7 $\pm$ 0.1 vs. 3.6 $\pm$ 0.4, $p < 0.01$). Testes from animals 10 days of age did not differ between treatment and control groups (Grade 2.8 $\pm$ 0.4 vs. 2.1 $\pm$ 0.3).

Direct *in vivo* evaluation of Müllerian duct regression in male fetuses by assessment of the urogenital ridge on gestational days 14, 15 and 16 after injection on day 13, revealed no difference between LHRH antiserum treated and controls. Müllerian inhibiting substance activity was Grade 0 in both groups of 14-day fetal testes, and not significantly different in 15-day fetal testes, Grade 2.4 $\pm$ 0.1 vs. 2.4 $\pm$ 0.2, and in 16-day fetal testes, 4.3 $\pm$ 0.2 vs. 4.0 $\pm$ 0.4 (LHRH antiserum vs. normal rabbit serum treated, respectively).

Testicular Weights and Penis Size. The weight of one testis was reduced in pups from dams injected with LHRH antiserum vs. that from dams given normal rabbit serum (Table 1). Similarly, the penis length was reduced in the same experimental groups. Body weights in experimental and control groups did not differ, inferring that general health was unaltered.

DISCUSSION

The antiserum to LHRH used here has been shown previously to act transiently and depress serum testosterone in adult rats for less than 7 days (5). LHRH antibody appeared to cross the maternal placental barrier because the male pups of antiserum injected mothers had smaller testes than those of control dams. Presumably, this was due to inhibition of secretion of one or both fetal gonadotropins. Furthermore, the testes of pups whose mothers had been injected with the antiserum to LHRH displayed increased secretion of Müllerian inhibiting substance in the organ culture bioassay. These studies suggest that one or both fetal gonadotropins inhibit the secretion of Müllerian inhibiting substance in late fetal and early neonatal life. The gonadotropin more likely to be responsible for this effect is FSH because FSH is known to control certain functions of the sertoli cell (11) and there is evidence that the sertoli cell is the source of Müllerian inhibiting substance (6, 13).

The lack of difference between experimental and control groups in the *in vivo* examination of the male urogenital ridge at 14-16 days is explicable. Any increase in the rate of Müllerian duct regression cannot be observed in less than 72 hours, the time interval necessary to visualize microscopically the morphological changes induced by Müllerian inhibiting substance, namely the autodigestion of the tissues (7, 21). Thus, one would expect to find no difference at this early time between Müllerian duct regression of the control and the experimental group. The *in vitro* bioassay results suggest that the content or release of Müllerian inhibiting substance from the testis is increased in animals whose endogenous serum gonadotropins are reduced. At 10 days of age there was no difference in Müllerian inhibiting substance activity in the testes suggesting a loss of sensitivity to gonadotropins, particularly FSH. This experiment eliminates the possibility of a stimulatory effect of gonadotropins on Müllerian inhibiting substance secretion.

Maternal injection of antiserum to LHRH also caused a marked decrease in the size of the penis of male pups. This suggests that the normally low circulating level of serum testosterone is necessary for normal penile growth.

This study indicates that normal Müllerian inhibiting substance secretion is dependent on hypothalamic function through LHRH regulation of gonadotropin release. Whether one or both gonadotropins are important should be studied following the administration of specific antisera to luteinizing hormone and follicle stimulating hormone.

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22. Received for publication May 11, 1977.

23. Accepted for publication September 21, 1977.

ACKNOWLEDGEMENTS

The authors thank Drs. Seymour Reichlin, Jack Crawford, Ineze Beitins, and Fabrizio Michelassi for their helpful suggestions. We also thank Mr. Carmelo Bondi and Thomas Manganaro for technical assistance and Ms. B.J. Capistran, Ms. Margaret Doherty and Mrs. Penny Colbert for secretarial assistance.

Figure 1

Schematic representation of experimental protocol. Luteinizing hormone releasing hormone antiserum (LHRH-AS) or normal rabbit serum (NRS) were injected into pregnant dams at gestational ages 13 and 20 days and into pups at 6 days postnatal life. Groups of rat testes from fetuses were assayed for Müllerian inhibiting substance (MIS) activity at 17 days' fetal age and from pups at 6 and 10 days postnatal life.

Figure 2

Müllerian inhibiting substance activity of testes of fetuses of dams treated with either luteinizing hormone releasing hormone (LHRH) antiserum or normal rabbit serum. The means $\pm$ SE of the graded organ culture assay are compared in 17-day fetuses, 6 and 10-day old rats.

Table 1. Testis weight, penis length, and body weight in rats born to dams treated with luteinizing hormone releasing hormone antiserum (LHRH-AS) or normal rabbit serum (NRS)

Treatment	Testis weight (mg)	Penis length (mm)	Body weight (g)
6 days old:			
LHRH-AS	5.8 $\pm$ 0.3 *	5.69 $\pm$ 0.17 **	10.1 $\pm$ 0.3
NRS	7.0 $\pm$ 0.3	6.50 $\pm$ 0.16	10.0 $\pm$ 0.4
10 days old:			
LHRH-AS	7.9 $\pm$ 0.3 **	5.93 $\pm$ 0.07 ***	10.9 $\pm$ 0.4
NRS	10.9 $\pm$ 0.7	6.84 $\pm$ 0.05	11.4 $\pm$ 0.4

Each mean $\pm$ SE represents 5 to 9 animals * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Figure 3

Müllerian duct regression caused by testes from 17-day fetuses and 6-day old male rats born to dams treated with luteinizing hormone releasing

hormone (LHRH) antiserum or normal rabbit serum. Arrow points to Müllerian duct in each section.

0031-3998/78/0012-0139\$02.00/0

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