BIOLOGY OF REPRODUCTION 15, 329–334 (1976)

The Production of Mullerian Inhibiting Substance by the Fetal, Neonatal and Adult Rat[1]

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ABSTRACT

The secretory pattern of Mullerian Inhibiting Substance in the rat was documented by a graded organ culture assay on each day of the perinatal period and at more widely spaced intervals in the adult period. The influence of placenta and pituitary on Mullerian Inhibiting Substance production was studied. Fetal testicular fragments were displaced from target Mullerian ducts to determine if Mullerian Inhibiting Substance would act at a distance.

Rat testes produced Mullerian Inhibiting Substance throughout the latter third of gestation. Its production continued during the first three weeks of extrauterine life, after which it disappeared, coincident with weaning. Hypophysectomy carried out after 20 days of age was ineffective in restoring Mullerian Inhibiting Substance activity to the adult rat testis. Placental fragments neither prolonged nor enhanced the Mullerian Inhibiting Substance activity of perinatal testes in organ culture. Mullerian Inhibiting Substance exerted its regressive effect on Mullerian ducts placed at a distance from a testicular source.

INTRODUCTION

Mullerian Inhibiting Substance is known to be produced by the fetal testes of many species including rabbit (Jost, 1946a, b; 1947; Picon, 1971), rat (Picon, 1969, 1970), chicken (Maruad et al., 1966; Groenendijk-Huijbers et al., 1974), calf (Jost et al., 1973; Josso, 1972b, 1973), and human (Josso, 1971, 1972a), during the middle third of gestation, and to cause regression of the Mullerian duct during that important interval. The substance continues to be elaborated by the testes after the Mullerian duct has undergone regression (Groenendijk-Huijbers, 1974; Maraud et al., 1966) but its function during the post regression stage is unknown. Since a sensitive assay for the detection of Mullerian Inhibiting Substance is available (Donahoe et al., 1976), it seemed appropriate to study the pattern of synthesis of Mullerian Inhibiting Substance by the testes during the post regression stage in order to gain some understanding of the determinants of its production and to furnish some clues regarding other possible functions that the substance may subserve. This report describes a detailed investigation of the intensity and duration of Mullerian Inhibiting Substance secretion by the fetal, newborn, and adult rat testes and the effect of the placenta and pituitary on this secretory pattern, both *in vitro* and *in vivo*.

It is not clear whether this fetal regressor can act at a distance in a humoral fashion. Therefore, the response of the Mullerian duct to Mullerian Inhibiting Substance produced by testicular fragments placed at a distance from the ducts was studied using a graded organ culture assay (Donahoe et al., 1976). Our studies confirmed the conclusions of the elegant vitteline membrane experiments of Josso, but avoided the potential of diapedesis of cells through contiguous membranes of large pore size. Documentation of the humoral action of Mullerian Inhibiting Substance is essential to determining the direction of biochemical isolation procedures.

MATERIALS AND METHODS

The presence of Mullerian Inhibiting Substance was assayed by a graded organ culture method (Donahoe et al., 1976). Briefly, the agonadal reproductive ducts from 14½ day female fetal rats were placed on an agar

Accepted May 17, 1976.
Received March 2, 1976.
[1] This work was supported by the Hood Foundation, and the National Cancer Institute #CA17393.
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coated stainless steel grid of a Falcon 3010 organ culture dish. Testicular tissue to be assayed was cut in 1–2mm fragments and placed adjacent to the ducts in the position normally occupied by the ovary, with the Mullerian duct lateral to the Wolffian duct. A 6-0 suture placed perpendicular to the cephalic end of the ducts facilitated orientation during histologic preparation. Incubations were performed at 37°C for 72 h in a humidified atmosphere of 95 percent air and 5 percent CO_2. Specimens were then fixed in buffered formalin, dehydrated in an alcohol series, cleared in xylene, and embedded in paraffin. The craniel end of the duct only was then 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 V.

Assay Material

Fetal rat testis. Testes harvested from fetuses of all ages from 14 to 22 days of gestation were incubated with the indifferent ducts of the 14½ day female fetal rat to study fetal elaboration of Mullerian Inhibiting Substance during the middle and later thirds of gestation.

Postnatal rat testis. Fresh testicular fragments obtained from rats 1 to 28 days after birth were incubated with the indifferent ducts for three days. Testicular fragments from the same postnatal testes were incubated also with rat placenta obtained when the fetal specimens were taken. Placental tissue was added so that the testicular fragments lay between the placental tissue and the indifferent ducts. Placenta alone was incubated with ducts as a control. Mullerian Inhibiting Substance activity was studied in testicular tissue specimens taken on almost every postnatal day of the first month. Also testis samples were obtained at more widely spaced intervals from adult animals ranging from 30 to 240 days of age. Freshly harvested adult and fetal ovary, adrenal, liver, muscle and placenta served as controls.

Hypophysectomized rat testis. Males hypophysectomized at 20, 40 and 60 days of age were obtained from Hormone Assay (Chicago) and shipped 10 days after surgery to assure both survival from surgery and development of the hypophysectomized state. Small fragments of testicular tissue freshly harvested between 10 and 30 days after hypophysectomy were assayed with the indifferent ducts.

Male rat amniotic fluid. Sterile, amniotic fluid from 17 day male fetuses was pooled and placed in the wells of the organ culture dish. Fourteen day female indifferent ducts were incubated with maternal muscle on the agar coated grid above the amniotic fluid. The Mullerian duct was then examined histologically for regression. Homogenates of normal adult testes and the testes of hypophysectomized animals were tested in a similar manner.

Distance Studies

A) Rat fetal testicular fragments were incubated at varying concentric distances from the indifferent target ducts (Fig. 1) and regression was graded.

B) Small fragments of maternal muscle (Fig. 2) or agar measuring 1–2mm were used to separate female 14 day indifferent ducts from the 14 day male urogenital ridge (testes, Mullerian and Wolffian duct).

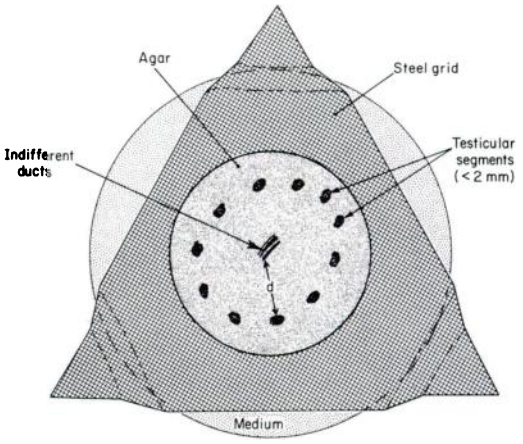

FIG. 1. Fetal testicular fragments placed at increasing distances from the Mullerian and Wolffian ducts of the 14 day female fetal rat.

After three days of incubation, regression of the Mullerian ducts from both male and female fetuses was evaluated.

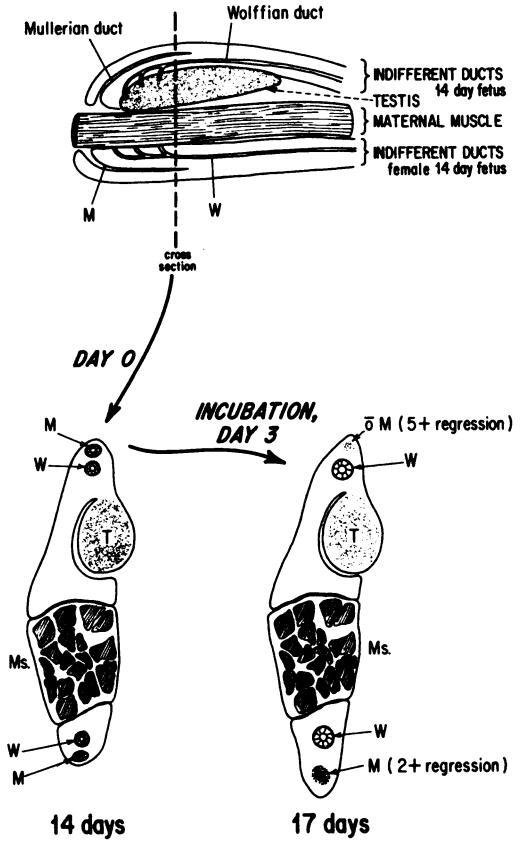

FIG. 2. Fourteen day fetal male ducts and testis separated from 14 day fetal female ducts by a 1–2mm muscle buffer.

TABLE 1. Regression of Mullerian duct performed by fetal rat testes.

Fetal rat day of gestation	Number of incubations	Number of regressions
14	21	21
15	4	4
16	4	4
17	13	13
18	8	8
19	16	16
20	15	15
21	14	14
22	3	3

RESULTS

Fetal Rat Testis

Testis of the fetal rat from 14 to 22 days gestation invariably caused marked (Grade V) regression of the Mullerian duct of the 14½ day female fetus with very little loss of activity during the latter third of gestation as the fetuses neared term (Table 1).

Postnatal Rat Testis

Mullerian Inhibiting Substance activity was still high during the first week of extrauterine life but gradually decreased after Day 4 (Fig. 3). Activity still could be detected readily during the second and third week of extrauterine life. After 21 days Mullerian Inhibiting Substance could no longer be detected by this assay. Testes, and testes with placenta, demonstrated a highly significant linear regression from Day 0 to Day 20 after birth. Testes alone had a correlation coefficient of 0.409 with a significance level of $P<0.005$. Testes with placenta had a correlation coefficient of 0.465 with a significance level of $P<0.001$. There is little significant difference between the two as judged by paired *t* test. Therefore, placenta had no detectable effect on prolonging the *in vitro* regressive activity of Mullerian Inhibiting Substance. After 21 days, Mullerian Inhibiting Substance could no longer be detected from either whole tissues or homogenates of adult testes. All assays of testicular tissues of animals from 27 to 240 days of age had no detectable Mullerian Inhibiting Substance activity.

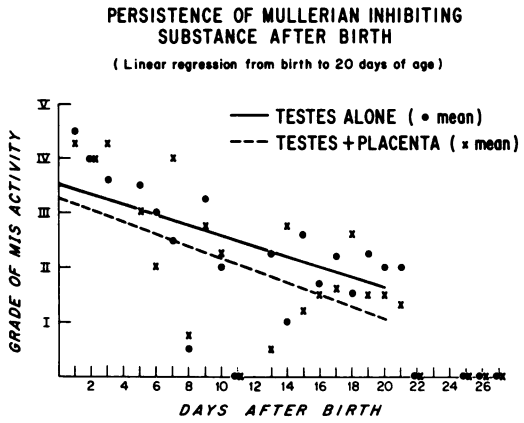

FIG. 3. Persistence of Mullerian Inhibiting Substance after birth. There is a linear regression of activity from birth to 20 days in the rat. Each point (• or X) represents the mean of 4 experiments.

Nontesticular Tissue

Mullerian Inhibiting Substance is highly specific to the testes. Except for an insignificantly small number of regressions (2/32) with placental fragments, where duct injury was suspected as the cause of the positive results, no other fetal or adult rat tissue (ovary, adrenal, muscle and liver) caused significant regression of the Mullerian ducts. All other placental fragments allowed excellent Mullerian duct development (Fig. 4).

Hypophysectomized Rat Testis

Testes of animals hypophysectomized at 20, 40 and 60 days of age, and harvested between 10 and 30 days later, consistently failed to cause regression of Mullerian ducts (Table 2). Further, homogenates and ultrafiltrates of the testes of hypophysectomized rats caused no regression of Mullerian ducts.

Male Rat Amniotic Fluid

Pooled, sterile, fluid collected from the amniotic sacs of male 17 day fetal rats failed to cause regression of the Mullerian ducts.

Distance Studies

Testicular fragments placed closer than 3mm to the indifferent ducts (Fig. 1) caused regression of the Mullerian ducts. Those placed further away than 3mm failed to cause regression. Fragments placed closer than 2mm often were found bunched together on top of the indifferent ducts, having been pulled in by capillary attraction. Indifferent ducts were

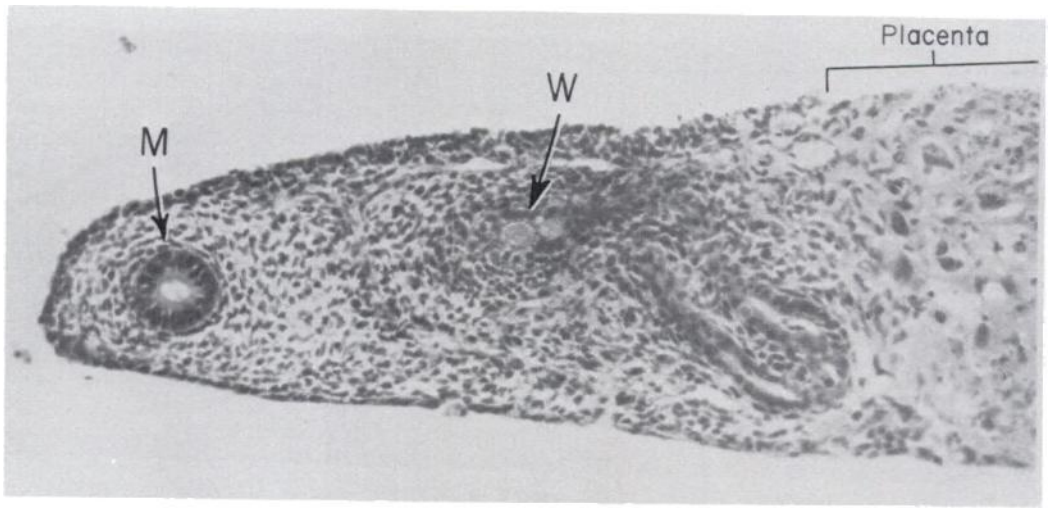

FIG. 4. Incubation of Mullerian duct with placental tissue. No regression is evident.

therefore separated from the 14 day testes by small 1–2mm blocks of agar. Regression was observed, but the ducts often curled, making sectioning and interpretation difficult. Therefore, small blocks of muscle were interposed between the ducts and fetal testes. The ducts remained straight for sectioning, and regression was observed consistently (Fig. 2).

DISCUSSION

Josso demonstrated a decline in the activity of Mullerian Inhibiting Substance during the latter part of gestation in bovine (Josso, 1973) and human (Josso, 1972c) testes. This report, like Picon's (1969, 1970, 1971) demonstrated that the fetal rat testes continues to elaborate Mullerian Inhibiting Substance from Day 14 to the end of gestation, after all *in vivo* regression is complete. Studies to ascertain when in fetal life the substance is first detectable have not been reported. Picon (1970) and Constantinople and Walsh (1973), showed that Mullerian Inhibiting Substance in the rat is inconstantly detectable for an undefined period after birth. This report confirms their findings, but precisely describes the duration and intensity of Mullerian Inhibiting Substance activity as the newborn rat matures (Fig. 3). Production is high during the first week of life (Grade III and IV), gradually falls off (Grade I and II) during the second and third week of extra-uterine life, and, after 21 days, is undetectable by this organ culture technique. Cessation of activity coincided with weaning. This may indicate that Mullerian Inhibiting Substance is controlled either by some maternal product transmitted by mother's milk or by maturation of the blood testis barrier which occurs at that time (Dym et al., 1970).

It is not known whether Mullerian Inhibiting Substance is produced autonomously or is under endocrine influence. Cessation of its production at birth in large mammals or at the weaning age in the rat led us to explore indirectly the role of the placenta and the pituitary in its control. Pituitary inhibition of testicular production of Mullerian Inhibiting

TABLE 2. Mullerian duct preservation with testes of hypophysectomized rats.

Day of hypophysectomy	Number of incubations: Number of days after hypophysectomy 10	20	30	Number of regressions
20	55	73	19	0
40	5	7	3	0
60	6	...	...	0

Substance in the young chicken was hypothesized by Maraud et al. (1969, 1970). They (Maraud et al., 1966) were able to restore activity by hypophysectomizing chickens at one month of age. Assay of the testes at two months of age was positive. They speculated that the maturing hypophysis may exert an inhibitory effect on the testicular production of Mullerian Inhibiting Substance. In contrast, we were unable to restore Mullerian Inhibiting Substance activity to the testes of rats hypophysectomized after 20 days, the age of weaning, and the earliest age at which survival from the surgical procedure can be expected. When fragments of placenta from 14 day pregnancies were incubated with the testes of postnatal rats from Day 1 to Day 28, the placental fragments failed to increase or extend the production of Mullerian Inhibiting Substance by the testicular fragments beyond that seen with testicular fragments alone. This may indicate that the placenta does not enhance the testicular production of Mullerian Inhibiting Substance, or that the timing of addition of placenta was inappropriate to effect a change. Nonetheless, because of the coincidence of loss of Mullerian Inhibiting Substance activity was weaning, prolactin, the principal placental hormone of the rat, deserves particular future attention, since it may have a stimulatory effect on the testicular production of Mullerian Inhibiting Substance.

Mullerian Inhibiting Substance could not be detected in the pooled amniotic fluid of 17 day male fetuses, indicating either that the substance is inactivated during transport to the amniotic fluid or that the bioassay lacks the sensitivity to detect it in unconcentrated form.

It is commonly believed that testicular tissue must be in direct contact with the target organ to produce regression. However the regression of Mullerian structures that occurs in the female calf of heterosexual bovine twin pregnancies suggests that Mullerian Inhibiting Substance must be transferred humorally from the male calf (Jost et al., 1972; Lillie, 1916) to produce the Freemartin state in its female twin. The results of the distance studies of this communication, in which testicular fragments were separated from the Mullerian duct, support the conclusions of the membrane studies of Josso (1972a) and provide additional evidence that the substance is secreted, exerts its effect across a distance, and does not require cell mediation except possibly by way of the indifferent mesenchyme surrounding the duct cells. It has also been possible to demonstrate (Swann et al., 1976) that all cell free extracts and purified fractions obtained from active testes cause Mullerian duct regression in the organ culture assay. These studies provide additional evidence that the substance(s) responsible for the observed phenomenon do not require cell to cell contact.

ACKNOWLEDGMENT

The authors acknowledge helpful discussions and continued interest of John F. Enders, John D. Crawford, David A. Swann, and J. Micheal Price.

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