Use of monoclonal antibody techniques to study the ontogeny of bovine anti-Müllerian hormone

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Summary. Monoclonal antibodies against bovine anti-Müllerian hormone (AMH) were used to study the hormone in cattle. Anti-Müllerian activity of testicular tissue, immunoreactive testicular AMH, serum AMH concentration and AMH production by incubated testicular tissue were detectable from 42 days, i.e. at the time of seminiferous tubule differentiation, and peaked between 50 and 80 days, when the Müllerian ducts regress in the male fetus. All the values stabilized at a lower level until 30 days after birth and then slowly decreased. At 18 months, only traces of AMH immunoreactivity were detectable in testicular tissue and serum concentration and AMH production by incubated testicular tissue were negligible; the main source of AMH in the adult animal was the rete testis fluid. Study of the disappearance rate of AMH from the serum of castrated calves gave a half-life of approximately 2 days for bovine AMH.

Introduction

Anti-Müllerian hormone (AMH), a glycoprotein produced by immature Sertoli cells, is responsible for regression of the Müllerian ducts in male fetuses (Jost, 1947). However, testicular AMH production is not restricted to the period of physiological Müllerian duct regression: until the onset of pubertal maturation, testicular tissue induces partial regression of fetal Müllerian ducts in organ culture (Picon, 1970) and, even in the adult, some anti-Müllerian activity can be recovered from rete testis fluid (RTF) (Josso, Picard, Dacheux & Courot, 1979). These data have been obtained through an in-vitro bioassay based upon the response of AMH of rat fetal Müllerian ducts (Picon, 1969). Following the development of monoclonal antibodies to bovine AMH (Vigier, Picard & Josso, 1982b) other techniques, such as immunocytochemistry and radioimmunoassay, have become available. These new methods have been used to evaluate the ontogeny of bovine AMH.

Materials and Methods

Animals. With the exception of fetuses over 80 days, all animals were bred at the Institut National de la Recherche Agronomique, and their age was known exactly (see Table 1). Younger fetuses were obtained after superovulation (Mauléon, Mariana, Benoit, Solari & Chupin, 1970), or blastocyst transfer (Renard, Ozil & Heyman, 1981). The sex of fetuses < 44 days was determined by cytogenetic analysis (Vigier, Prépın & Jost, 1972). Fetuses from cows at 80–270 days of gestation

were obtained at a slaughterhouse, and their ages were estimated from crown-rump lengths (Maneely, 1952). All fetuses were transported to the laboratory under refrigeration, within 4–6 h after slaughter.

Table 1. Age, sex and gestational status of the animals used in the study

Age	Males		Females
	Singletons	Co-twins to freemartins	
Fetuses (days <i>p.c.</i>)			
38–74	17	10	7
80–240	29	15	6
Calves (days <i>post partum</i>)			
8–316	35	—	5
Bulls (1.5–5 years)			
Intact	7	—	
Vasectomized	6	—	

Methods. Each animal was studied by one or several of the following methods. Bioassay for anti-Müllerian activity of testicular tissue was carried out in 35 animals according to Picon (1969) and the results were expressed as indicated by Josso, Forest & Picard (1975). Usually, testicular tissue from one fetus or post-natal bull was co-cultured with 4–6 fetal rat reproductive tracts, but fetuses <44 days yielded an amount of tissue sufficient for association with only 1 or 2 tracts. Light microscopy immunochemistry was performed in 29 cases as previously described (Tran & Josso, 1982), except that the fixative described by McLean & Nakane (1974) was employed, and the avidin-biotin-peroxidase complex technique was used according to Hsu, Raine & Fanger (1981). The biotinylated horse immunoglobulin directed against mouse immunoglobulin and the avidin-biotin-peroxidase complex were purchased from Vector Laboratories, Burlingame, California, U.S.A.

Serum AMH was measured by a radioimmunoassay (Vigier, Legeai, Picard & Josso, 1982a) in intact animals of various ages, and in 3 calves at various times after castration. Each sample was assayed in quadruplicate, except for some samples of fetuses <80 days, from which there was only enough serum for 2 determinations. AMH was also measured by RIA in rete testis fluid (RTF) collected at slaughter of 18-month- and 5-year-old bulls, in seminal plasma of 2-year-old bulls and in testicular tissue incubation medium. The medium consisted of Eagle's Minimum Essential Medium, with added penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). Incubation conditions were adapted to the amount of available tissue. Testicular tissue obtained from animals >165 days *post coitum* (*p.c.*) was minced and 100 mg amounts were distributed into 3 vials, each containing 2 ml incubation medium. The vials were incubated for 1 h at 37°C in a Dubnoff metabolic shaker, under a 95% O₂ + 5% CO₂ atmosphere, then centrifuged for 5 min at 200 g. The medium was discarded, replaced by 2 ml fresh medium, and incubation resumed for 2 h. After centrifugation for 10 min at 2300 g the tissue was discarded and the medium frozen until assay. In fetuses <165 days, this convenient and accurate procedure was not feasible, and an alternative one was devised. Testicular tissue was weighed under sterile conditions, and 1–15 mg were cut into small fragments, which were placed upon an agar-coated grid, in a dish to which 0.4–0.8 ml Eagle's MEM, fortified with 10% female fetal calf serum, was added, up to the level of the grid. The dish was incubated at 37°C under a 95% air + 5% CO₂ atmosphere, for 24 h, and then the medium was aspirated, weighed and frozen. Results of incubation experiments are expressed as mU AMH produced per mg testicular tissue over 2 or 24 h. In 2 calves, aged 30 and 103 days, both incubation procedures were used: within 24 h, the amount of AMH released per mg

tissue was respectively 4.4 and 3.2 times that produced over 2 h. It follows that results obtained with the 24-h method should be divided by 4 to allow a rough comparison with the results given by the 2-h method.

Text-fig. 1. AMH concentrations in serum and rete testis fluid. No difference in AMH serum concentrations between singletons and male co-twins to freemartins was detected.

Text-fig. 2. AMH production by gonadal tissue *in vitro* measured over 2 or 24 h. The scales are adjusted to allow comparison between the 2 methods: experiments performed in duplicate showed that the amount of AMH produced by incubated tissue in 24 h is approximately 25% of that produced in organ culture during 24 h.

Table 2. Serum and testicular AMH in bulls of different ages

Age	Testicular anti-Müllerian activity (no. of rat reproductive tracts with Müllerian duct regression)			Testicular AMH localization*	AMH (mU produced/mg testicular tissue)		AMH conc. (mU/ml)	
	Complete	Incomplete	None		In 2 h	In 24 h	Serum	RTF
Fetuses (days <i>p.c.</i>)	0	0	2	0		0	0	
	0	2	0	0		0-0.16†	0	
				++		0.49		
	13	0	0	+++		2.25		
	30	7	0	++++		2.78 ± 1.18	19.01 ± 10.9	
	6	11	0	++++		1.7 ± 0.04	15.01 ± 8.37	
121-280	6	25	0	++++	0.47 ± 0.07	0.64 ± 0.17	9.13 ± 2.05	
Calves (days <i>post partum</i>)	5	15	1	++++	0.40	1.74	10.04 ± 1.56	
	0	10	3	++ to ++++	0.28 ± 0.05		7.72 ± 3.19	
				++	0.095	0.30	6.11 ± 2.68	
							3.23 ± 1.67	
Bulls	Intact	0	0	4	0.001 ± 0.001†		†0.53 ± 0.39	3.7 ± 1.2
							†0.17 ± 0.03	—‡
	Vasectomized	2					0	3.42
		2					†0.18 ± 0.03	
	3						0	

Values are mean ± s.d.
* Intensity of reaction to immunocytochemical localization.
† Values computed from the standard curve beyond the limits of its published validation.
‡ AMH concentrations in the seminal plasma of 2 animals were 0.8 and 3.0 mU/ml.

Results

The bioassay results for anti-Müllerian activity and for immunocytochemical localization of AMH in testicular tissue of fetuses and post-natal bulls are given in Table 2, together with the mean serum AMH concentration and the mean production of AMH by incubated testicular tissue in different age groups. Individual values are given in Text-fig. 1 for serum and RTF concentrations and in Text-fig. 2 for AMH production.

The results obtained by the different methods are roughly comparable: AMH, first secreted at 42–43 days *p.c.*, was produced in large amounts between 50 and 80 days. In older fetuses, anti-Müllerian activity, AMH production by incubated tissue, and, slightly later, AMH serum concentration, stabilized at a lower level, which was maintained for up to 1 month after birth. After that time a slow, progressive, decrease was observed for all the characteristics of AMH biosynthesis studied. At 18 months, anti-Müllerian bioactivity of testicular tissue was no longer detectable and only traces of AMH immunoreactivity remained (Pl. 1, Figs 1 & 2). Serum concentration and AMH release by incubated testicular tissue were no longer accurately measurable by RIA, but were still significantly different from 0. At 5 years, AMH was no longer detectable in the serum of intact or vasectomized bulls but was still present in RTF. The rate of disappearance of immunoreactive AMH from the serum of calves after castration is shown in Text-fig. 3.

Text-fig. 3. Decrease of serum AMH following castration of 1-month-old bulls ($r = -0.98$). The half life of AMH ($T_{1/2}$) is approximately 2 days.

Discussion

The results of the present study confirm and extend those obtained by Picon (1970) for the rat, and by other investigators for other species (Donahoe, Ito, Price & Hendren, 1977a; Tran, Meusy-Dessolle & Josso, 1981), showing that AMH is essentially a product of the fetal testis and that its synthesis decreases with the progression of testicular maturation. Various methodological approaches have allowed us to gain some measure of insight into the mechanisms responsible for this chronological evolution.

The start of AMH production by the bovine testis at 42–43 days *p.c.* coincides with the formation of seminiferous tubules (Vigier *et al.*, 1976), a chronological relationship already recognized in the pig (Tran *et al.*, 1977) and in keeping with the Sertoli cell origin of the hormone (Blanchard & Josso, 1974; Tran & Josso, 1982). The peak production of AMH between 50 and 80 days *p.c.* corresponds to the period of physiological regression of Müllerian ducts in the male calf (Vigier *et al.*, 1976), and serum AMH levels remain elevated up to 120 days. A similar correlation

between testosterone-dependent steps of sexual differentiation and testosterone concentration in fetal serum and testicular tissue has been observed in the human fetus by Winter, Faiman & Reyes (1977), who also noted a time lag between testicular and serum androgen levels. By contrast, our observation that testicular anti-Müllerian activity is highest during the period of Müllerian regression in the calf is not consistent with findings previously reported in the rat. Müllerian duct regression is more or less completed by 18 days *p.c.* in the male rat fetus (Picon, 1969) yet rat fetal testes retain maximal anti-Müllerian activity up to the time of birth (Picon, 1970; Donahoe, Ito & Hendren, 1977b). Rat and bovine AMH molecules are not identical, since monoclonal antibodies raised against one do not necessarily recognize the other (Vigier *et al.*, 1982b). Possibly, the rat Müllerian duct, which serves as target organ for the AMH of both species, is more responsive to the homospecific hormone. In that case, maximal regression of rat Müllerian ducts could occur *in vitro* under the influence of testicular tissue from older rat fetuses, even if it no longer produces maximal amounts of AMH. Accurate measurement of AMH production by the rat testis must await the production of antibodies recognizing murine AMH.

The progressive fall of AMH biosynthesis in calves > 1 month of age is in keeping with the decrease in anti-Müllerian activity previously reported by Donahoe *et al.* (1977a). These investigators could no longer detect bioactivity in testes from calves over 10 weeks, in contrast to our own findings at 3 months of age. However, the discrepancy is slight and could be due to breed differences or to a mistaken evaluation of the age of the animals obtained at a slaughterhouse by the Boston investigators. Using sensitive immunochemical techniques, we were able to detect AMH in the serum and testicular incubation medium of young adult bulls, but, at that age, RTF was the most abundant source of AMH, a finding which tallies with the demonstration of a low amount of anti-Müllerian bioactivity in pig RTF (Josso *et al.*, 1979).

AMH is produced by Sertoli cells and the rate of its biosynthesis and/or release into the peripheral circulation is affected not only by the level of production by individual Sertoli cells, but also by the total number of Sertoli cells and by the membrane permeability of seminiferous tubules at a given stage of testicular maturation. After birth, mitotic activity of Sertoli cells is repressed, while germ cells proliferate (Erickson, 1964; Curtis & Amann, 1981). The ensuing dilution of Sertoli cells within testicular tissue has been used to explain the decrease of androgen-binding protein concentration in the testis of developing lambs, at a time when the rate of synthesis of the protein by individual cells does, in fact, increase (Carreau, Drosowsky & Courot, 1979). Sertoli cell dilution could similarly contribute to the fall of anti-Müllerian activity of testicular explants and decreased production of AMH per unit volume of incubated tissue. On the other hand, AMH serum concentration is positively correlated with the ratio between total number of active Sertoli cells and blood volume. Even given a stable production of AMH per Sertoli cell, one would expect AMH serum concentration to decrease in bulls > 6 months of age in which the vascular space continues to increase (Abdel-Raouf, 1961), while the number of Sertoli cells per testis remains constant (Attal & Courot, 1963). Formation of the blood-testis barrier at 6–7 months (Curtis & Amann, 1981) further contributes to the cut-off of the AMH supply to the peripheral blood. Establishment of the blood-testis barrier in the rat is followed by a fall in serum concentration of androgen-binding protein (Gunsalus, Musto & Bardin, 1978).

PLATE 1

Immunocytochemical localization of bovine AMH. Cryostat sections (10 µm) were stained by the avidin-biotin-peroxidase technique. × 135.

Fig. 1. The reaction for AMH in a 4-month-old fetal calf testis is intense and limited to Sertoli cell cytoplasm. Germ cells and Sertoli cell nuclei are unstained.

Fig. 2. The reaction in a 1.5-year-old bull testis is very faint in Sertoli cytoplasmic processes.

PLATE 1

(Facing p. 212)

For AMH, however, Sertoli cell dilution and establishment of the blood–testis barrier are not the main causes of waning AMH production and serum concentration. Only traces of immunoreactive AMH persist in adult Sertoli cells (Pl. 1, Fig. 1), but such cells are heavily stained by an immunocytochemical reaction carried out with an antibody specifically directed against androgen-binding protein (Pelliniemi *et al.*, 1981; Attramadal, Bardin, Gunsalus, Musto & Hansson, 1981).

In conclusion, this study shows that bovine AMH is essentially a product of fetal Sertoli cells, and that its period of maximal production coincides with its physiological effect in sex differentiation, namely regression of fetal Müllerian ducts. Whether the residual secretion of AMH by adult Sertoli cells has any physiological significance remains to be determined.

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