

Anti-Müllerian hormone and freemartinism : inhibition of germ cell development and induction of seminiferous cord-like structures in rat fetal ovaries exposed *in vitro* to purified bovine AMH (1)

B. VIGIER, F. WATRIN, S. MAGRE (*), D. TRAN, O. GARRIGOU, M. G. FOREST (**), N. JOSSO

*Unité de Recherches sur l'Endocrinologie du Développement, INSERM U.293
Hôpital des Enfants-Malades, 75743 Paris cedex 15, France.*

() Laboratoire de Physiologie du Développement, Collège de France,
5, place Marcelin-Berthelot, 75005 Paris Cedex 15.*

*(**) Unité de Recherches : Endocrinologie et Développement de l'Enfant, INSERM U.34
Hôpital Debrousse, 69005 Lyon, France.*

Summary. In 13 and 14-day old fetal rat ovaries maintained 3 to 10 days in organ culture, purified bovine anti-Müllerian hormone (AMH) (1.5 to 3 µg/ml) induced a characteristic freemartin effect. Gonadal volume and germ cell number were significantly reduced, compared to control ovaries cultured in an hormonal medium, and epithelial cells with large clear cytoplasm linked by interdigitations differentiated in the gonadal blastema. These cells resembling rat fetal Sertoli cells became polarized and formed seminiferous cord-like structures delineated by a basal membrane containing laminin and fibronectin as is the case of testicular seminiferous cords at the first step of their differentiation. These data indicate that AMH is probably the testicular factor responsible for the morphological modifications of bovine freemartin gonads and suggest that this hormone could also be involved in normal morphological differentiation of the testis. In contrast, in fetal rat ovaries, AMH did not trigger the testosterone production which occurs in freemartin gonads at an early stage of the gestation.

Introduction.

Factors controlling the differentiation of mammalian fetal reproductive tracts are now well known. As first indicated by Jost in 1953 in male fetuses, the maintenance of Wolffian ducts is due to testosterone secreted by fetal Leydig cells, whereas anti-Müllerian hormone (AMH), a glycoprotein dimer (Picard *et al.*, 1978), secreted by immature Sertoli cells (Blanchard and Josso, 1974), is

(1) Part of this work has been previously published (Vigier *et al.*, 1987).

responsible for Müllerian duct regression. In contrast, the factors controlling gonadal differentiation and germ cell development are still unknown. When the Müllerian ducts in bovine female « freemartin » fetuses, attached to a male by chorionic vascular anastomoses, regress under the effect of AMH originating from the male testis (Vigier *et al.*, 1984b), the growth of the freemartin ovary is arrested and the germ cells cease to multiply. Then, seminiferous cord-like structures begin to develop in approximately half the cases (Jost *et al.*, 1972, 1973, 1975). To investigate the possibility that AMH might be the initial factor of ovarian abnormalities in bovine freemartin fetuses, as suggested by Jost *et al.* (1972, 1973) and Vigier *et al.* (1976), the prospective ovaries of 13-14-day old fetal rats were exposed to purified bovine AMH in organ culture.

The following experiments show that bovine AMH induced gonadal stunting, germ cell loss and differentiation of seminiferous cord-like structures in rat ovaries and that these effects were both time and dose-dependent.

Material and methods.

Bovine AMH (bAMH) was purified by immunochromatography on a monoclonal antibody as previously described (Picard and Josso, 1984), and quantified by a competition-type liquid phase radioimmunoassay (Vigier *et al.*, 1985).

TABLE 1

Number of fetal rat ovaries studied after various periods of culture in the presence of bovine AMH.

Culture period (days pc)	bAMH (µg/ml)	Number of gonads studied by		
		LM	EM	ICC
13-17	0	4	1	
	3	4	1	
13-19	0	7	1	
	1.5	5		
	3	13	1	
14-17	0	3		
	3	3		
14-19	0	16	13	
	0.75	4		2
	1.125	4		
	1.5	4	1	2
	1.875	2	1	
	3	8	9	2
14-2*	0	5	2	4
	3	6	1	3
	1.5	3		4
20-3*	0	5		
	3	5		
3*-8*	0	4		
	3	4		

LM: light microscopy; EM: electron microscopy; ICC: immunocytochemistry; pc: post-coïtum; * days post-partum.

Ovarian culture. — Ovaries were removed under a dissecting microscope from fetal and, on one occasion, neonatal Wistar rats at various periods of development and cultured for various periods of time, as specified on table 1. Ovaries were explanted alone, except in cultures initiated at 13-14 days post-coitum (p.c.), where they were explanted with adjacent mesonephros and genital ducts. Fetal age was assessed as previously described (Magre and Jost, 1984) with coitus assumed to take place at 1 AM. Cultures were always initiated and terminated between 2 and 6 PM; however, for simplicity's sake, the extra 13-17 hr period has been disregarded and fetal age expressed as an integer. Thirteen and fourteen-day old fetuses were sexed by a sex chromatin test performed on a fragment of the amniotic membrane (Jost, 1972). The ovarian explants were placed on agar-coated grids in organ-culture dishes (Falcon). Culture medium (1066 CMRL, Eurobio France), either with or without AMH and supplemented with bovine serum albumin, 0.5 %, penicillin 100 UI/ml, streptomycin 100 µg/ml, was added up to the level of the grid and renewed every 2 or 3 days. The culture dishes were incubated for periods stated in table 1, at 36 ± 1 °C, in a 95 % air, 5 % CO² atmosphere. In addition, 14-day old fetal testes and 15-day-old fetal testes and ovaries were cultured 5 or 6 days in control medium and in the presence of 3 µg/ml of bAMH, respectively, for morphological study and testosterone assay.

Histological processing. — The explants to be examined by light microscopy were fixed in Bouin's fluid for 24 hrs, dehydrated, embedded in paraffin and serially sectioned at 5 µm. One section out of five was mounted and stained with Ehrlich's hematoxylin and eosin. Specimens intended for electron microscopy were fixed overnight at 4 °C in glutaraldehyde 2.5 %, paraformaldehyde 2 % in sodium cacodylate buffer 0.05 M, pH 7, rinsed and postfixed 1 hr in 2 % osmium tetroxide in the same buffer. They were then dehydrated, embedded in Epon and semi-thin and thin sections were obtained at various levels of the gonad.

Assessment of germ cell number and gonadal size. — Germ cells in various stages of the meiotic prophase were identified according to their nuclear characteristics (Beaumont and Mandl, 1962; Bézard and Mauléon, 1984) and counted on serial 5-µm sections, one out of 5 for those ovaries obtained or explanted at 13-14 days p.c., and one out of 20 for the other ones. The total number of germ cells present in the ovary was generated by multiplying the number of germ cells counted on each section by 5 or 20 as appropriate. To determine the volume of the gonads, an outline of the sections separated by 25 µm was drawn after projection, and the surface determined with an image analyzer. The volume between two sections was considered to be a 25-µm high cylinder with a base equal to the mean of the surfaces of the two sections.

Immunohistochemical procedures. — Rat fetal ovaries cultured 5 or 10 days in the presence or absence of AMH were tested for AMH immunoreactivity as previously described (Tran *et al.*, 1987). Cultured fetal ovaries were also tested for fibronectin and laminin deposition as described by Agelopoulou and Magre (1987), using antisera to human plasma fibronectin and to mouse laminin obtained from Bethesda Research Laboratories (Herblay, France).

Testosterone radio-immunoassay was performed, according to the technique described by Forest *et al.* (1973), in the culture medium of 15 day-old fetal testis and ovaries maintained 6 days in the presence or absence of AMH.

Statistical analysis. — The mean germ cell number in different treatment groups was compared by analysis of variance, followed by a Student's t-test when appropriate. Student's t-test was used to compare the gonadal volume in control and AMH-exposed ovaries.

Results.

Effect of bAMH upon germ cell number of cultured fetal ovaries. — The evolution of germ cell number in developing rat ovaries *in vivo* and *in vitro*, in the presence or absence of AMH, is shown on figure 1. *In vivo*, the number of germ

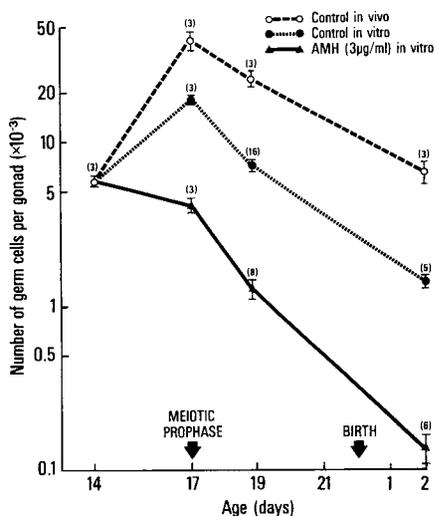


FIG. 1. — *Number of germ cells in developing rat ovaries in vivo and in vitro.* *In vivo*, the number of germ cells increases markedly between 14 and 17 days and then decreases, when meiotic maturation is initiated. Control cultured ovaries show roughly the same pattern, but the number of germ cells is lower at each time-point. In contrast, in ovaries exposed *in vitro* to AMH (3 µg/ml) germ cells do not proliferate and their number diminishes already at 17 days.

cells increased sharply between 14 and 17 days p.c. and then steadily decreased. In control cultured ovaries, at each time point, the number of germ cells was inferior to that noted *in vivo*, but the evolutionary trend was similar. In contrast, the germ cell population in fetal rat ovaries cultured in the presence of AMH

(3 $\mu\text{g}/\text{ml}$) did not increase between 14 and 17 days, and after 17 days the rate of germ cell degeneration was similar in AMH-treated and control ovaries. These data indicate that AMH acted essentially by inhibiting germ cell proliferation, and not by accelerating germ cell degeneration.

The effect of increasing doses of AMH upon number of germ cells in 14-day-old fetal rat ovaries cultured 5 days is shown on figure 2 by comparison with control cultures. AMH, at a concentration of 3 $\mu\text{g}/\text{ml}$, decreased germ cell number by 82 %, and the threshold concentration of AMH for this effect is approximately 1 $\mu\text{g}/\text{ml}$. Ovaries explanted a day earlier (fig. 3) exhibited a lower survival of germ cells in control culture ($p < 0.001$) and also an increased sensitivity to AMH, only 10 % withstanding a 1.5 $\mu\text{g}/\text{ml}$ concentration of the hormone versus 50 % in ovaries explanted at 14 days (fig. 2).

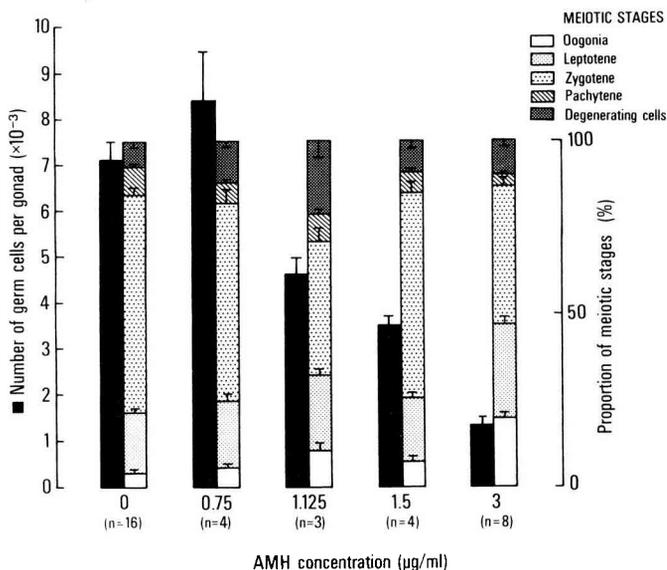


FIG. 2. — Effect of increasing concentrations of purified bovine AMH upon number and meiotic maturation of germ cells in 14-day-old fetal rat ovaries cultured 5 days. AMH reduces the total number of germ cells per fetal ovary in a dose-dependent manner. The threshold concentration of AMH is between 0.75 and 1.125 $\mu\text{g}/\text{ml}$. In contrast, the progression of meiotic prophase maturation was affected only at the highest dose used (see text).

The influence of the culture period upon the number of germ cells in control and AMH-treated cultures is shown on figure 4. In cultures terminated at 17 (fig. 4A) instead of 19 days, more germ cells survived in both control and AMH-treated cultures, but the 23 % proportion of AMH-resistant germ cells was quite similar to the 18 % proportion observed in ovary cultures up to 19 days (fig. 2). Lengthening the culture period up to 24 days (fig. 4B) dramatically

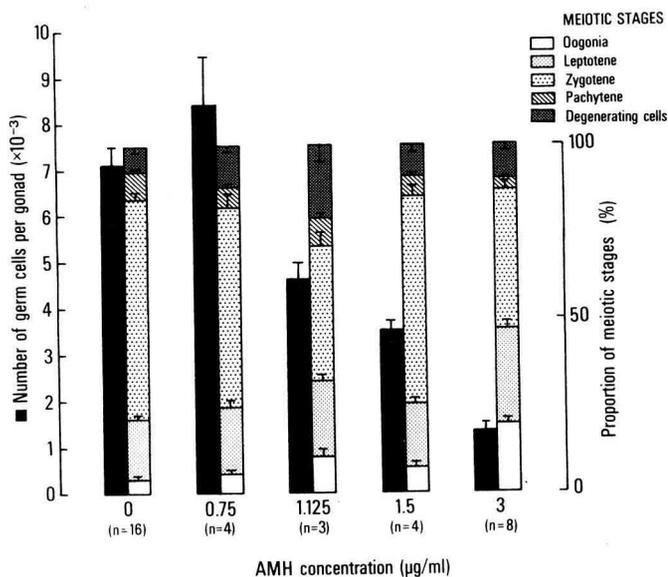


FIG. 3. — Effect of purified bovine AMH upon number and meiotic maturation of germ cells in fetal rat ovaries cultured from 13 to 19 days post-coitum. Bovine AMH dramatically reduces the number of surviving germ cells and retards the progression of meiotic maturation, as shown by the increased proportion of oogonia in AMH-treated cultures.

decreased the number of surviving germ cells in the control cultures, but 11 % still resisted AMH. Finally, AMH had no effect upon the number of ovarian germ cells in cultures initiated at 20 days (fig. 4C) or upon male germ cells : the total number of germ cells in testes cultured from 14 to 19 days was $11,445 \pm 2,043$ in controls and $14,023 \pm 2,578$ in cultures exposed to 3 µg/ml AMH.

Influence of AMH upon meiotic prophase maturation. — In cultures performed from 14 to 19 days (fig. 2), AMH had no effect upon the distribution of cells in various stages of meiotic prophase except at the higher dose employed (3 µg/ml). At this concentration the percentages of oogonia and leptotene were increased but the absolute number was similar in AMH-treated and control cultures, due essentially to the selective loss of germ cells in advanced stages of meiosis in AMH-treated ovaries.

In contrast, AMH did retard meiotic maturation in cultures initiated at 13 days (fig. 3) or in 14-day ovaries cultured up to 24 days (fig. 4B) ; in both cases, the absolute number of oogonia and germ cells in the initial stage of meiotic prophase was significantly higher in AMH-treated than in control ovaries, and few germ cells developed beyond the pachytene stage in ovaries cultured 10 days in presence of AMH whereas most had reached the dyctyate stage in the control medium.

AMH had no effect on meiotic maturation in ovaries explanted later at 20 days p.c. (fig. 4C).

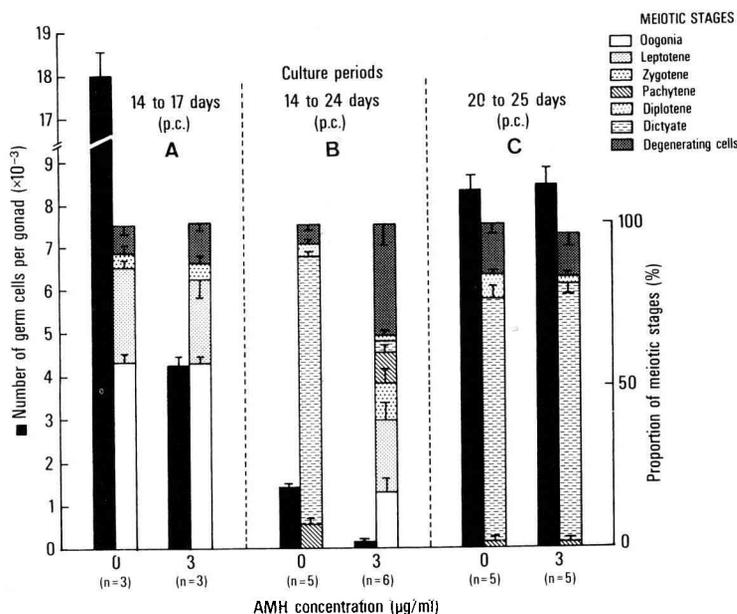
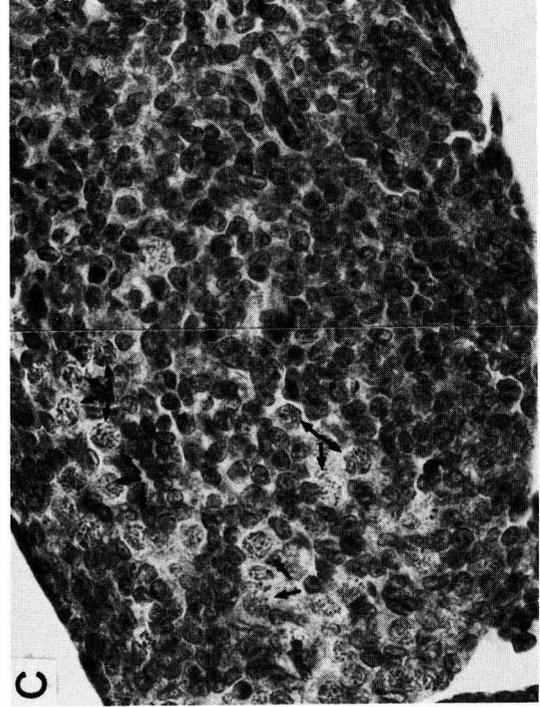
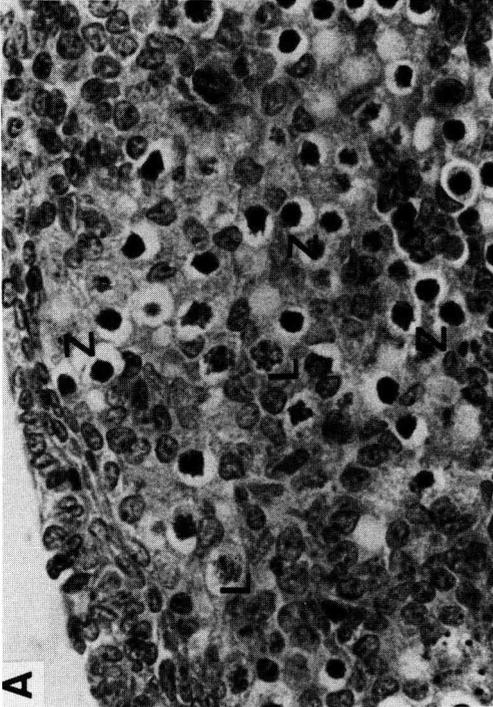
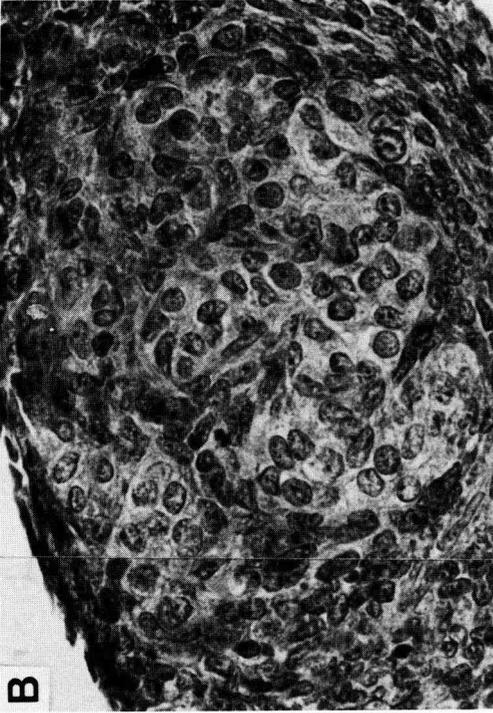


FIG. 4. — Effect of the culture period upon the response of fetal rat ovaries to bovine AMH. A) 14-day-old ovaries cultured 3 days: in AMH-treated cultures the number of germ cells is reduced by 77%. Progression of meiotic maturation is not significantly affected. B) 14-day-old ovaries, cultured 10 days. In AMH-treated cultures, germ cell number is reduced by 89%, cell degeneration is increased, and meiotic maturation is retarded. Oogonia and leptotene stages absent in control culture are still observed in the presence of AMH. C) 20-day-old ovaries cultured 5 days. AMH has no effect, either upon germ cell number or upon the progression of meiotic prophase maturation.

Gonadal volume. — AMH significantly reduced gonadal volume. In 14-day-old fetal ovaries cultured 5 days, mean gonadal volume \pm SEM was $0.1016 \pm 0.0044 \text{ mm}^3$ in controls and 0.0375 ± 0.0073 in ovaries exposed to 3 $\mu\text{g}/\text{ml}$ AMH ($p < 0.001$).

Gonadal structure. — In all 14-day-old ovaries cultured 5 days in the presence of AMH at a concentration of 1.5 $\mu\text{g}/\text{ml}$ or over (fig. 5B), differentiating cord-like structures containing somatic cells of epithelioid appearance were irregularly disseminated in the gonadal blastema. At the surface of the gonad, a layer of flattened connective cells resembled a differentiating tunica albuginea. Ultrastructural examination indicated that groups of epithelial cells developed in the gonadal blastema, forming cord-like structures resembling differentiating seminiferous tubules (fig. 6B). These epithelial cells, joined by typical interdigitations, acquired a globular nucleus and an enlarged cytoplasm, less electron-dense than that of other cell types. The amount of rough endoplasmic reticulum did not increase as is the case of fetal testicular Sertoli cells.



Control cultures, performed in the absence of AMH, were characterized by an homogeneous blastema containing numerous germ cells in leptotene and zygotene stages, located in nests separated by somatic cells of mesenchymal aspect (fig. 5A).

In cultures maintained 10 days, AMH-induced structural modifications were enhanced (fig. 5D). Cord-like structures were more frequent and conspicuous. They could be distinguished from authentic seminiferous tubules of 13 or 14-day old testes only by the fact that they contained few germ cells. The epithelial cells were polarised along a basement membrane which contained dense deposits of laminin and fibronectin (fig. 7A-C). In control ovaries (fig. 5C), ovarian follicles had not yet developed, and germ cells in the diplotene or dichtyate stages were disseminated in the gonadal blastema. Immunohistochemical staining revealed a tendency towards compartmentalization of the blastema by laminin deposits (fig. 7B), but these were relatively faint and discontinuous, and did not surround discrete cords, as in the AMH-treated ovaries. In contrast to these, control ovaries contained little or no fibronectin (fig. 7D).

In cultures initiated at 20 days p.c. and later, AMH did not induce structural modifications nor interfere with folliculogenesis. In ovaries explanted at 3 days p.p. and maintained 5 days in culture, the development of numerous follicles was well advanced in presence or absence of AMH (results not shown).

Tests for endogenous AMH production. — Within the limits of sensitivity of our immunohistochemical method, no AMH immunoreactivity was detected in 14-day-old fetal ovaries after 5 or 10 days of AMH treatment.

Test for testosterone production. — No testosterone secretion was observed in the culture medium of 15-day-old ovaries maintained 6 days in presence or absence of AMH. In contrast, the amount of testosterone secreted per fetal testis over 3 days of culture in similar conditions reached 3.6 to 5.8 ng (table 2).

Müllerian ducts regression witnessed by narrowing of the lumen, epithelial disorganization, and the formation of a ring of connective tissue around the epithelium occurred in a dose-dependent manner in all cultures exposed from 13 or 14 days onwards to a 0.75 to 1.125 $\mu\text{g}/\text{ml}$ concentration of AMH. At AMH concentrations of 1.5 $\mu\text{g}/\text{ml}$ or over, the Müllerian duct had totally disappeared. In all control cultures, Müllerian ducts were normal (results not shown).

FIG. 5. — *Effect of AMH upon histological structure of 14-day-old ovaries.* A) Ovary cultured 5 days in control medium. Note numerous germ cells in leptotene (L) or zygotene (Z) meiotic prophase. B) Developing seminiferous cord-like structures containing somatic epithelial-like cells in an ovary cultured 5 days in the presence of 3 $\mu\text{g}/\text{ml}$ AMH. Few germ cells are detectable on this section. C) Ovary cultured 10 days in control medium. Germ cells in pachytene or diplotene stage (arrows) dispersed in an undifferentiated blastema. D) Note conspicuous seminiferous cord-like structures delineated by a basement membrane lined by small fusiform cells in ovary cultured 10 days in the presence of 1.5 $\mu\text{g}/\text{ml}$ AMH. All sections are stained by hematoxylin-eosin; AB \times 500; CD \times 430.

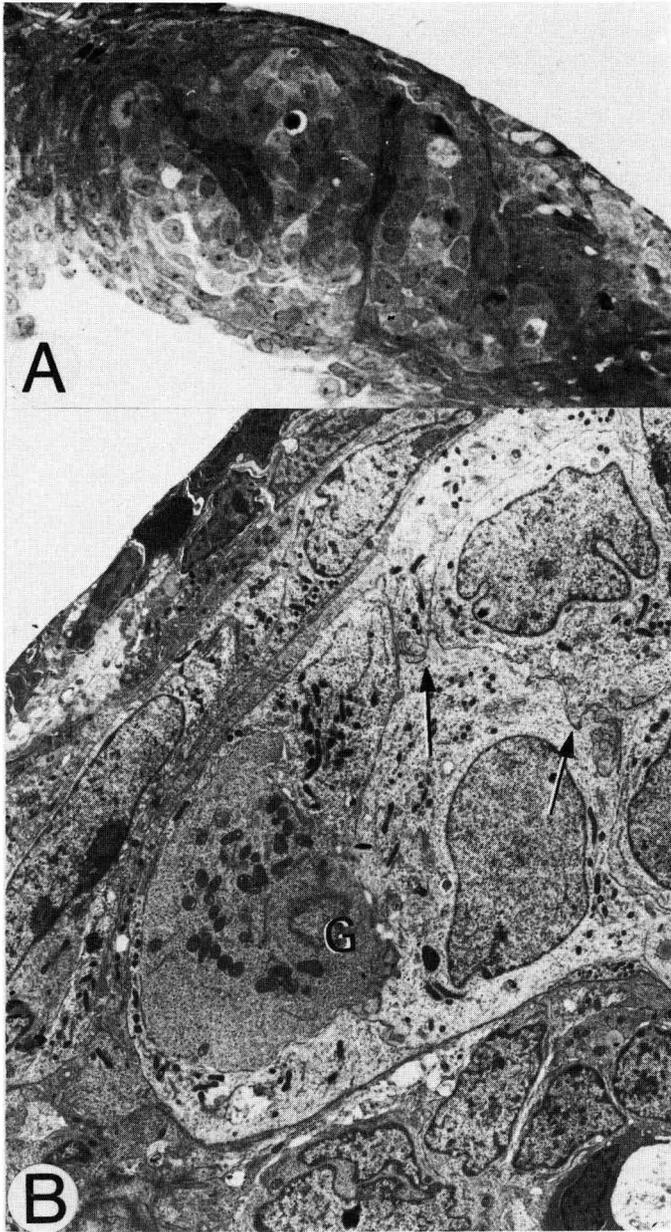
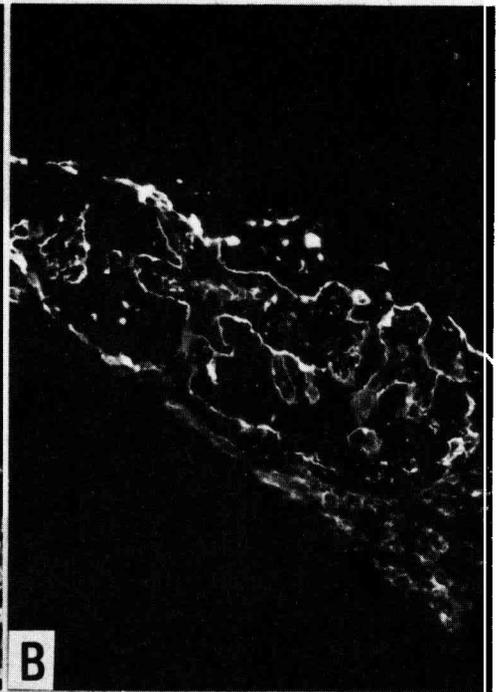
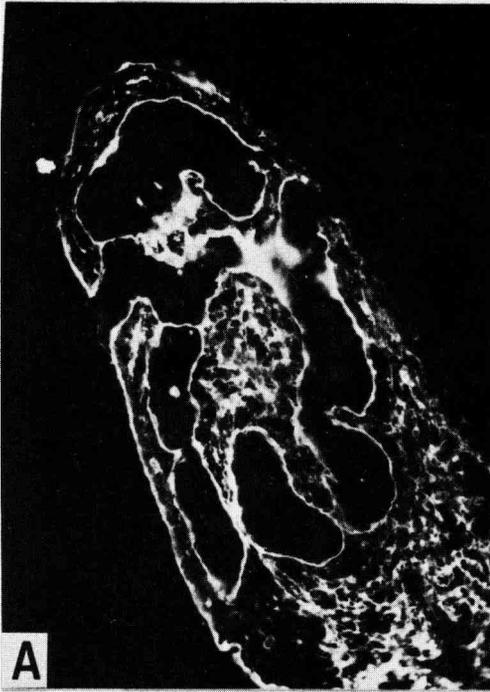


FIG. 6. — *Seminiferous cord-like structures in the anterior pole of a 14-day-old fetal rat ovary cultured 5 days in the presence of AMH (3 $\mu\text{g}/\text{ml}$).* A) Semi-thin section showing seminiferous cord-like structures separated by dense fusiform mesenchymal cells. $\times 300$. B) Electron micrograph showing epithelial-like cells, with abundant, clear cytoplasm, joined by interdigitations (arrows), and grouped in cord-like structures. These cells resemble developing Sertoli cells, but lack prominent rough endoplasmic reticulum. Outside the cords and at the surface of the gonad connective tissue is differentiating. $\times 3\,280$. G : germ cell.

AMH (3 μ g/ml)

CONTROLS

LAMININ



FIBRONECTIN

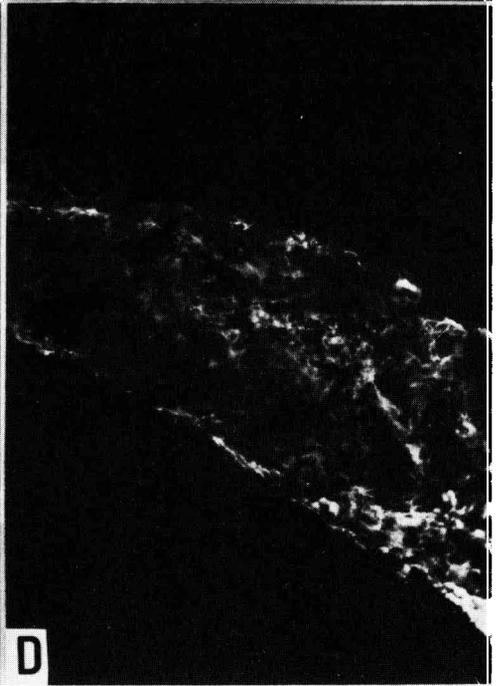
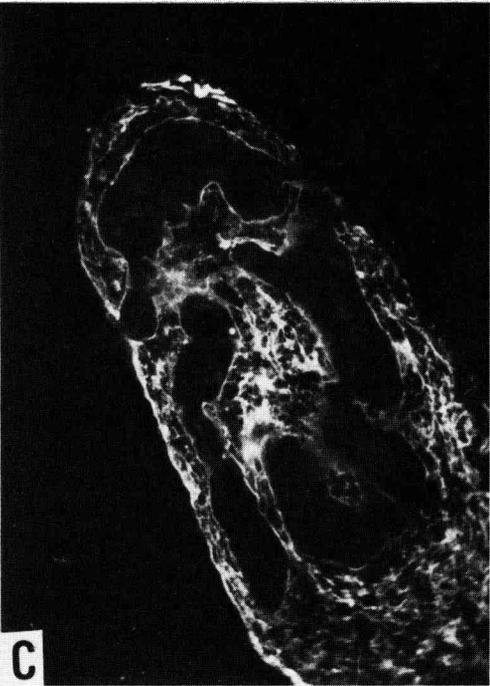


FIG. 7. — Immunohistochemical localization of fibronectin and laminin in 14-day-old fetal rat ovaries cultured 10 days in the presence (A and C) or absence (B and D) of AMH (3 μ g/ml). Note the heavy deposit of laminin and, to a lesser degree, of fibronectin around the seminiferous cord-like structures induced by AMH treatment. \times 250.

TABLE 2

Testosterone secretion by 15 day old fetal rat gonads in organ culture.

Gonads	Culture medium	Number of cultures (1)	T (ng/gonad) over culture period	
			day 0-3	day 3-6
Ovaries	Control	2	0.021 ± 0.014	0.029 ± 0.021
	AMH (1.5-2.25 µg)	2	0.017 ± 0.020	0.027 ± 0.024
Testes	Control	7	5.852 ± 1.696	3.568 ± 1.877
	AMH (1.5-2.25 µg)	8	5.789 ± 0.558	4.084 ± 2.735
None	Control	3	0.005 ± 0.006	
	AMH (1.5-2.25 µg)	3	0.006 ± 0.006	

(1) 5 to 8 gonads in each culture.

T = Testosterone (mean ± SD).

Discussion.

To investigate the effect of bovine AMH upon fetal ovary we used 13-14-day-old fetal rat gonads in organ culture because this model is reliable and well documented. The results obtained on the development and meiosis maturation of germ cells in control anhormonal medium were comparable to those reported previously in the same species *in vitro* by Rivelis *et al.* (1976) and Prépin *et al.*, (1985a and b) and *in vivo* by Beaumont and Mandl (1962) and Bézard and Mauléon (1984). The development of the fetal rat ovary is characterized by an initial stage of active oogonial replication up to 17 days p.c., at which point the germ cells enter the meiotic prophase and many fall victims to waves of degeneration. *In vitro*, the evolution of meiotic maturation was identical to that observed *in vivo* with approximately a 24-hr lag (Rivelis *et al.*, 1976). Our results show that AMH interfered essentially with oogonial replication, whereas after entry in meiotic prophase, the rate of germ cell loss was similar in control and AMH-treated cultures (fig. 1). Prépin *et al.* (1985a) also found that the decrease in ovarian germ cell population elicited by co-culture with fetal testicular tissue was due to inhibition of oogonial replication. AMH did not prevent germ cells from entering meiosis, but delayed this process in ovaries explanted at 13 days after 5 days in culture, and in ovaries explanted at 14 days and cultured 10 days. In these conditions, some oogonia were still present, and a few germ cells had reached late stages of meiotic prophase (fig. 3-4B). The similarity of these results with the freemartin situation is striking. At the time AMH secretion is initiated by the bovine testis of the male twin, germ cells in the prospective ovary of the fetal freemartins no longer multiply. Some of them fail to enter meiosis and none develop beyond the zygotene or, at best, pachytene stage (Jost *et al.*, 1975; Prépin *et al.*, 1979).

In addition to its inhibiting effect upon germ cell development, AMH induced the formation of seminiferous cord-like structures in ovaries exposed to it in culture. AMH-treated ovaries developed structures lined by epithelial cells, delineated by a basement membrane containing laminin and fibronectin. Fibro-

nectin appeared very early around differentiating seminiferous tubules, and may play a role in the polarization of Sertoli cells (Paranko *et al.*, 1983). The epithelial cells contained in these structures exhibited some characteristics of developing Sertoli cells, namely globular nuclei, clear, abundant cytoplasm and interdigitations (Magre and Jost, 1980). Their rough endoplasmic reticulum however was not enlarged, and this may perhaps explain why we were unable to detect the presence in these cells of immunoreactive AMH, which is localized in this region of the fetal Sertoli cell (Tran and Josso, 1982 ; Hayashi *et al.*, 1984). Furthermore, AMH is not easily detected in the very early stages of testicular differentiation, since only one out of four 13-day-old rat fetal testes yielded positive results with this technique (Tran *et al.*, 1987). Seminiferous tubules containing immunoreactive AMH (Vigier *et al.*, 1984b) develop only in approximately half the freemartin gonads after 90 days (Jost *et al.*, 1973, 1975), and their rate of AMH secretion is very low (Vigier *et al.*, 1984b).

A large body of literature deals with efforts to reproduce the freemartin model experimentally by transplanting, grafting or culturing testes with fetal ovaries. Conflicting results have been reported. The bewildering variety of ovarian modifications obtained can be explained by differences in the species and protocol used. In general, fetal testes have been found more effective ovarian transformers than adult ones. Severe growth inhibition of ovaries and germ cells, and the occasional development of seminiferous cord-like structures are the most frequent modifications observed (see for review Prépin *et al.*, 1985a ; Taketo *et al.*, 1985 ; Vigier *et al.*, 1987).

The nature of the fetal testicular factor capable of modifying ovarian organogenesis has been the subject of much speculation. In the freemartin, a diffusible substance is apparently involved (Vigier *et al.*, 1976). Ohno *et al.*, (1976) and Wachtel *et al.* (1980) have suggested that H-Y antigen is the culprit, but no conclusive evidence has been obtained in favor of the testis-inducing role of this gene product (Mc Laren *et al.*, 1984). Few conspicuous modifications have been observed *in vitro* in young fetal rat ovaries cultured in presence of H-Y antigen secreted by Daudi cell teratoma (Benhaim *et al.*, 1982). Other investigators (Jost *et al.*, 1972 ; Vigier *et al.*, 1977 ; Stoll *et al.*, 1980 ; Rashedi *et al.*, 1983), struck by the similar timing of ovarian lesions and Müllerian regression, have proposed that both are mediated by the same substance, namely AMH. Using AMH purified by immunochromatography on a monoclonal antibody (Picard and Josso, 1984), we are now in a position to confirm this hypothesis. In our studies, AMH added to culture medium, consistently reduced germ cell number and induced the formation of seminiferous cord-like structures containing polarized epithelial cells resembling fetal Sertoli cells. These findings cannot be attributed to germ cell depletion, which is known to interfere with the formation of follicles but does not induce the formation of testicular cords (Merchant, 1975 ; Merchant-Larios and Centeno, 1981). Furthermore, some of these cord-like structures contained a few germ cells (fig. 6B). AMH had no effect upon fetal ovaries at day 20, and did not perturb the formation of ovarian follicles, indicating that a critical phase of sensitivity to AMH, previously demonstrated for Müllerian ducts (Picon, 1969), can also be delineated for germ

cells. In the ovary, this critical phase was terminated at the time of differentiation of granulosa cells, the cells which produce AMH in the ovary (Vigier *et al.*, 1984a). Bovine AMH had no effect upon male germ cells, proving that its effect upon young fetal ovaries was not due to non-specific toxicity.

In bovine freemartin, Shore and Shemesh (1981) have shown that 47-130-day-old fetal gonads were able to produce significant amounts of testosterone *in vitro* (0.16 to 2.3 ng/gonad/24 h). In 15-day fetal rat ovaries, AMH was unable to induce testosterone production but this negative result could be due to species differences. In bovine ovaries, steroidogenesis characterized by the transitory secretion of estradiol takes place very early at the time of fetal gonadal differentiation (Shore and Shemesh, 1981), whereas in rat ovaries this process occurs only after birth (Picon *et al.*, 1985).

Among the other questions raised by this study, the possibility that AMH might play a role in normal testicular differentiation should be considered. Can AMH be viewed as an autocrine agent promoting terminal differentiation of its producer cell type? Obviously, AMH cannot be the primary factor responsible for testicular differentiation, since biosynthesis of AMH by Sertoli cells implicates prior differentiation of this cell lineage. However, it is conceivable that AMH might be required at some critical point (after the triggering of testicular organogenesis through some other Y-directed stimulus) to regulate germ cell proliferation, complete Sertoli cell differentiation, and assist seminiferous cord edification.

*Reçu en octobre 1987.
Accepté en avril 1988.*

Acknowledgements. — We are grateful to Dr Jacques Prépin and to Jacqueline Bézard for advice on recognizing meiotic phases, to Odette Locquet, Marthe Solvar, Jacqueline Campargue and Marie-Pierre Monneret for devoted technical assistance, to Jan Berghège for photographic processing and to Catherine Dalleu for typing the manuscript. The work was supported by a grant from I.N.R.A. : ATP « Hormones hypophysaires et fonction de reproduction chez les Vertébrés ».

Résumé. *Hormone anti-Müllérienne et freemartinisme : inhibition du développement des cellules germinales et induction de cordons de type testiculaire dans les ovaires de fœtus de rat cultivés en présence d'AMH bovine purifiée.*

L'AMH bovine purifiée a provoqué dans les ovaires de fœtus de rat de 13-14 jours, maintenus 3 à 10 jours en culture organotypique, un effet freemartin caractéristique. Le volume de la gonade et le nombre de cellules germinales sont réduits de façon significative par rapport aux ébauches ovariennes cultivées en milieu témoin an hormonal. De plus, l'AMH a induit dans le blastème gonadique, la différenciation de cellules épithéliales à large cytoplasme clair aux électrons, unies entre elles par des interdigitations caractéristiques. Ces cellules qui ressemblent à des cellules de Sertoli fœtales se polarisent progressivement pour former des structures analogues à des ébauches de cordons séminifères délimitées par une membrane basale renfermant de la fibronectine et de la laminine, comme c'est le cas pour les cordons séminifères dès les premiers stades de leur différenciation. Ces faits indiquent que l'AMH est probablement le facteur testiculaire responsable des anomalies morphologiques de la gonade des freemartins bovins et suggèrent que cette hormone pourrait être impliquée également dans la différenciation

morphologique testiculaire normale. Par contre, l'AMH n'a pas déclenché, dans les ovaires des fœtus de rat, de production de testostérone qui a lieu, dans les gonades des fœtus freemartins, à un stade précoce de la gestation.

References

- AGELOPOULOU R., MAGRE S., 1987. Expression of fibronectin and laminin in fetal male gonads *in vivo* and *in vitro* with or without testicular morphogenesis. *Cell Diff.*, **21**, 31-36.
- BEAUMONT H. M., MANDL A. M., 1962. A quantitative and cytological study of oogonia and oocytes in the fetal and neo-natal rat. *Proc. roy. Soc. B*, **155**, 557-579.
- BENHAIM A., GANGNEREAU M. N., BETTANE-CASANOVA M., FELLOUS M., PICON R., 1982. Effects of H-Y antigens on morphologic and endocrine differentiation of gonads in mammals. *Differentiation*, **22**, 53-58.
- BLANCHARD M. G., JOSSO N., 1974. Source of the anti-Müllerian hormone synthesized by the fetal testis: Müllerian-inhibiting activity of fetal bovine Sertoli cells in tissue culture. *Pediatr. Res.*, **8**, 968-971.
- BÉZARD J., MAULÉON P., 1984. Evolution des cellules germinales femelles au cours de la prophase méiotique chez le rat: critères de reconnaissance cytoplasmique et nucléaire des différents stades en histologie fine. *Reprod. Nutr. Dévelop.*, **24**, 633-654.
- FOREST M. G., CATHIARD A. M., BERTRAND J. A., 1973. Total unbound testosterone levels in the newborn and in normal and hypogonadal children: use of a sensitive radioimmunoassay for testosterone. *J. clin. Endocr. Metab.*, **36**, 1132-1142.
- HAYASHI H., SHIMA H., HAYASHI K., TRELSTAD R. L., DONAHOE P. K., 1984. Immunocytochemical localization of Müllerian inhibiting substance in the rough endoplasmic reticulum and Golgi apparatus in Sertoli cells of the neonatal calf testis using a monoclonal antibody. *J. Histochem. Cytochem.*, **32**, 649-654.
- JOST A., 1953. Problems of fetal endocrinology: the gonadal and hypophyseal hormones. *Rec. Prog. Horm. Res.*, **8**, 379-418.
- JOST A., 1972. Données préliminaires sur les stades initiaux de la différenciation du testicule chez le rat. *Arch. Anat. micr. Morph. exp.*, **61**, 415-438.
- JOST A., PERCHELLET J. P., PRÉPIN J., VIGIER B., 1975. The prenatal development of bovine freemartins, 392-406. In REINBORN R., *Symposium on Intersexuality*, Springer-Verlag, Berlin.
- JOST A., VIGIER B., PRÉPIN J., 1972. Freemartins in cattle: the first steps of sexual organogenesis. *J. Reprod. Fert.*, **29**, 349-379.
- JOST A., VIGIER B., PRÉPIN J., PERCHELLET J. P., 1973. Studies on sex differentiation in mammals. *Rec. Progr. Horm. Res.*, **29**, 1-41.
- MAGRE S., JOST A., 1980. The initial phases of testicular organogenesis in the rat. An electron microscopy study. *Arch. Anat. micr. Morphol. exp.*, **69**, 297-318.
- MAGRE S., JOST A., 1984. Dissociation between testicular organogenesis and endocrine cytodifferentiation of Sertoli cells. *Proc. nat. Acad. Sci. USA*, **81**, 7831-7834.
- McLAREN A., SIMPSON E., TOMONARI K., CHANDLER P., HOGG H., 1984. Male sexual differentiation in mice lacking H-Y antigen. *Nature*, **312**, 552-555.
- MERCHANT H., 1975. Rat gonadal and ovarian organogenesis with and without germ cells. An ultrastructural study. *Develop. Biol.*, **44**, 1-21.
- MERCHANT-LARIOS H., CENTENO B., 1981. Morphogenesis of the ovary from the sterile W/Wv mouse, 383-392. In *Advances in the morphology of cells and tissues*, Alan R. Liss, New York.
- OHNO S., CHRISTIAN L. C., WACHTEL S. S., KOO G. C., 1976. Hormone-like role of H-Y antigen in bovine freemartin gonad. *Nature*, **261**, 597-598.
- PARANKO J., PELLINIEMI L. J., VAHERI A., FOIDART J. M., LAKKALA-PARANKO T., 1983. Morphogenesis and fibronectin in sexual differentiation of rat embryonic gonads. *Differentiation*, **23**, 372-381.
- PICART J. Y., JOSSO N., 1984. Purification of testicular anti-Müllerian hormone allowing direct

- visualization of the pure glycoprotein and determination of yield and purification factor. *Mol. cell. Endocrinol.*, **34**, 23-29.
- PICARD J. Y., TRAN D., JOSSO N., 1978. Biosynthesis of labelled anti-Müllerian hormone by fetal testes : evidence for the glycoprotein nature of the hormone and for its disulfide-bonded structure. *Mol. cell. Endocrinol.*, **12**, 17-30.
- PICON R., 1969. Action du testicule fœtal sur le développement *in vitro* des canaux de Müller chez le rat. *Arch. Anat. micr. Morph. exp.*, **58**, 1-19.
- PICON R., PELLOUX M. C., BENHAIM A., GLOAGUEN F., 1985. Conversion of androgen to estrogen by the rat fetal and neonatal female gonad : effects of dcAMP and FSH. *J. Steroid. Biochem.*, **23**, 995-1000.
- PRÉPIN J., CHARPENTIER G., JOST A., 1985a. Action du testicule fœtal sur le nombre des cellules germinales de l'ovaire du fœtus de rat *in vitro*. *C. R. Acad. Sci. (Paris) III*, **300**, 43-48.
- PRÉPIN J., GIBELLO-KERVAN C., CHARPENTIER G., JOST A., 1985b. Number of germ cells and meiotic prophase stages in fetal rat ovaries cultured *in vitro*. *J. Reprod. Fertil.*, **73**, 579-583.
- PRÉPIN J., VIGIER B., JOST A., 1979. Meiosis in fetal freemartin gonads and in rat fetal ovaries *in vitro*. *Ann. Biol. anim. Bioch. Biophys.*, **19**, 1263-1271.
- RASHEDI M., MARAUD R., STOLL R., 1983. Development of the testes in female domestic fowls submitted to an experimental sex reversal during embryonic life. *Biol. Reprod.*, **29**, 1221-1228.
- RIVELIS C., PRÉPIN J., VIGIER B., JOST A., 1976. Prophase méiotique dans les cellules germinales de l'ébauche ovarienne de rat cultivée *in vitro* en milieu an hormonal. *C. R. Acad. Sci. (Paris)*, **282**, 1429-1432.
- SHORE L., SHEMESH M., 1981. Altered steroidogenesis by the fetal bovine freemartin ovary. *J. Reprod. Fert.*, **63**, 309-314.
- STOLL R., RASHEDI M., MARAUD R., 1980. Hermaphroditism induced in the female chick by testicular grafts. *Gen. comp. Endocrinol.*, **41**, 66-75.
- TAKETO T., KOIDE S. S., MERCHANT-LARIOS H., 1985. Gonadal sex differentiation in mammals, 271-287. In *The origin and evolution of sex*, Alan R. Liss, New York.
- TRAN D., JOSSO N., 1982. Localization of anti-Müllerian hormone in the rough endoplasmic reticulum of the developing bovine Sertoli cell using immunocytochemistry with a monoclonal antibody. *Endocrinology*, **111**, 1562-1567.
- TRAN D., PICARD J. Y., CAMPARGUE J., JOSSO N., 1987. Immunocytochemical detection of anti-Müllerian hormone in Sertoli cells of various mammalian species, including man. *J. Histochem. Cytochem.*, **33**, 733-743.
- TURNER C. D., 1969. Experimental reversal of germ cells. *Embryologia*, **10**, 206-230.
- VIGIER B., LOCATELLI A., PRÉPIN J., Du MESNIL du BUISSON F., JOST A., 1976. Les premières manifestations du « freemartinisme » chez le fœtus de veau ne dépendent pas du chimérisme chromosomique XX/XY. *C. R. Acad. Sci. (Paris)*, **282**, 1355-1358.
- VIGIER B., PICARD J. Y., CAMPARGUE J., FOREST M. G., HEYMAN Y., JOSSO N., 1985. Secretion of anti-Müllerian hormone by immature bovine Sertoli cells in primary culture, studied by a competition type radio-immunoassay : lack of modulation by either FSH or testosterone. *Mol. cell. Endocrinol.*, **43**, 141-150.
- VIGIER B., PICARD J. Y., TRAN D., LEGEAI L., JOSSO N., 1984a. Production of anti-Müllerian hormone : another homology between Sertoli and granulosa cells. *Endocrinology*, **114**, 1315-1320.
- VIGIER B., PRÉPIN J., PERCHELLET J. P., JOST A., 1977. Développement de l'effet freemartin chez le fœtus de veau. *Ann. Méd. vét.*, **121**, 521-536.
- VIGIER B., TRAN D., LEGEAI L., BÉZARD J., JOSSO N., 1984b. Origin of anti-Müllerian hormone in bovine freemartin fetuses. *J. Reprod. Fert.*, **70**, 473-479.
- VIGIER B., WATRIN F., MAGRE S., TRAN D., JOSSO N., 1987. Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it *in vitro*. *Development*, **100**, 43-55.
- WACHTEL S. S., HALL J. L., MULLER U., CHAGANTI R. S. K., 1980. Serum-borne H-Y antigen in the fetal bovine freemartin. *Cell*, **21**, 917-926.