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Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it *in vitro*

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Summary

To determine whether anti-Müllerian hormone (AMH) is responsible for the gonadal lesions observed in bovine genetic females united by placental anastomoses to male twins (freemartins), prospective ovaries of fetal rats were exposed to purified bovine AMH *in vitro*. In cultures initiated at 14 days *p.c.* and maintained 3 to 10 days, AMH consistently induced a characteristic 'freemartin effect', namely reduction of gonadal volume, germ cell depletion and differentiation, in the gonadal blastema, of epithelial cells with large clear cytoplasm linked by interdigitations, resembling rat fetal Sertoli cells. These cells tend to become polarized and form cords, delineated by a continuous basal membrane containing laminin and fibronectin. Such structures, resembling developing seminiferous cords, were not detected in control ovarian cultures. These data strongly suggest that AMH is the testicular factor responsible for triggering the morphological abnormalities of freemartin gonads.

Key words: anti-Müllerian hormone, Müllerian-inhibiting substance, freemartin, gonadal differentiation.

Introduction

As initially proposed by Jost (1953), differentiation of fetal reproductive tract is controlled by two discrete testicular hormones. Testosterone produced by fetal Leydig cells is responsible for the maintenance and differentiation of masculine structures such as Wolffian duct derivatives. Anti-Müllerian hormone (AMH) also known as Müllerian-inhibiting substance (MIS) or factor (MIF), is a glycoprotein dimer (Picard, Tran & Josso, 1978; Picard & Josso, 1984; Picard, Goulut, Bourrillon & Josso, 1986), secreted by Sertoli cells (Blanchard & Josso, 1974; Tran & Josso, 1982; Hayashi, Shima, Hayashi, Trelstad & Donahoe, 1984), which causes the regression of the Müllerian ducts, which would otherwise give rise to female reproductive organs. In contrast, the factors controlling the development of germ cells and gonadal sex are not as clearly delineated. In mammals, gonadal differentiation is determined by the presence or absence of testis-determining genes on the Y chromosome, and environmental factors are generally thought to be incapable of overriding their influence (McLaren, 1985). Freemartinism (Lillie, 1917) is an exception to this rule. When heifers are united to a male twin by chorionic vascular anastomoses, their ovaries cease to grow, become depleted of germ cells and eventually, in approximately half the cases, develop seminiferous tubules (Jost, Vigier & Prépin, 1972; Jost, Vigier, Prépin & Perchellet, 1973; Jost, Perchellet, Prépin & Vigier, 1975). Müllerian derivatives regress at the same time in the freemartin and her male twin. The possibility that AMH, the testicular hormone responsible for Müllerian regression, might also mediate the ovarian changes observed in bovine freemartins has been considered (Jost *et al.* 1972, 1973; Vigier, Locatelli, Prépin, Du Mesnil du Buisson & Jost, 1976), but could not be investigated in the absence of purified AMH. We now show that purified bovine AMH induces stunting, germ cell loss and differentiation of seminiferous cord-like structures in fetal rat ovaries exposed to it in organ culture, and that these effects are both time and dose dependent.

Materials and methods

Bovine AMH

Bovine AMH (bAMH) was purified by immunochromatography on a monoclonal antibody as previously described (Picard & Josso, 1984) and quantified by a competition-type liquid-phase radioimmunoassay, using a specific polyclonal antibody (Vigier, Picard, Campargue, Forest, Heyman & Josso, 1985). One RIA unit corresponds to 3 μg pure protein (Picard *et al.* 1986). Bovine AMH was added at a final concentration of 0·75 to 3 $\mu g\,ml^{-1}$ to 1066 CMRL culture medium (Eurobio, France), containing penicillin 100 i.u. ml^{-1}, streptomycin 100 $\mu g\,ml^{-1}$, and bovine serum albumin 0·5 %. Culture medium was renewed every 2 or 3 days.

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 in Table 1. Ovaries were explanted alone, except in cultures initiated at 14 days, *post coitum* (*p.c.*), where they were explanted with adjacent mesonephros and genital ducts. Fetal age is assessed as previously described (Magre & Jost, 1984), with coitus assumed to take place at 1 a.m. Cultures were always initiated and terminated between 2 and 6 p.m., however, for simplicity's sake, the extra 13–17 h period will be disregarded and fetal age expressed as an integer. 14-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, either with or without AMH, was sterilized by passage through 0·22 μm filters (Millipore) and added up to the level of the grid. The culture dishes were incubated for periods stated in Table 1, at 36 ± 1 °C, in a 95 % air, 5 % CO_2 atmosphere. In addition, 14-day-old fetal testes were cultured 5 days, three in control medium, and three in the presence of 3 $\mu g\,ml^{-1}$ bAMH.

Histological processing

The explants to be examined by light microscopy were fixed in Bouin's fluid for 24 h, dehydrated, embedded in paraffin and serially sectioned at 5 μm. One section out of five was mounted and stained with Ehrlich's haematoxylin 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 h in 2 % osmium tetroxide in the same buffer. They were then dehydrated, embedded in Epon and semithin and thin sections were obtained at various levels of the gonad. Slides and grids were coded prior to histological examination.

Assessment of germ cell number and gonadal size

Germ cells, either in the resting phase or in various stages of the meiotic prophase were identified according to their nuclear characteristics (Beaumont & Mandl, 1962; Bézard & Mauléon, 1984) and counted on serial 5 μm sections, one out of five for those ovaries obtained or explanted at 14 days *p.c.*, and one out of twenty for other ones. The total

Table 1. *Number of fetal rat ovaries studied after various periods of culture in the presence of bovine AMH*

Culture period (days *p.c.**)	bAMH concentration ($\mu g\,ml^{-1}$)	Number of gonads studied by: light	electron microscopy	immunofluorescence
14 — 17	0	3		
	3	3		
14 — 19	0	16	13	
	0·75	4		
	1·125	4		
	1·5	4	1	
	1·875	2	1	
	3	8	9	
14 — 24 (2 *p.p.*†)	0	5	2	3
	3	6	1	3
	1·5	3		3
20 — 25 (3 *p.p.*)	0	5		
	3	5		
3 *p.p.* — 8 *p.p.*	0	4		
	3	4		

* *post coitum.*
† *post partum.*

number of germ cells present in the ovary, generated by multiplying the number of germ cells counted on each section by 5 or 20 as appropriate, was considered a reasonable approximation with, however, a slight risk of overestimation (Prépin, Gibello-Kervran, Charpentier & Jost, 1985*b*). To determine the volume of the gonads, an outline of sections separated by 25 μm was drawn after projection and the surface determined with an image analyser. The volume between two sections was considered to be a cylinder 25 μm high with a base equal to the mean of the surfaces of the two sections.

Immunohistochemical procedures

Testes from fetuses taken at 13 days + 18 h and 14 days + 8 h *p.c.*, and 14-day-old fetal ovaries cultured 5 or 10 days in the presence or absence of AMH were tested for AMH immunoreactivity as previously described (Josso, Tran, Picard & Vigier, 1986), except that biotin–streptavidin reagents (Amersham) were used. Controls were performed using either AMH-exhausted antibody or nonimmune rabbit IgG. Cultured fetal ovaries were also tested for fibronectin and laminin deposition as described by Agelopoulou & Magre (1986), using antisera to human plasma fibronectin, and to mouse laminin, obtained from Bethesda Research Laboratories (Herblay, France), and used at 1/100 dilution.

Statistical analysis

Comparison of the mean germ cell number in different treatment groups was performed 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 number of germ cells 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 in Fig. 1. *In vivo*, the number of germ cells increases sharply between 14 and 17 days *p.c.* and then steadily decreases. In control cultured ovaries, at each time point, the number of germ cells is inferior to that noted *in vivo*, but the evolutionary trend is similar. In contrast, fetal rat ovaries cultured in the presence of AMH, 3 $\mu g\,ml^{-1}$, do not increase their germ cell population between 14 and 17 days, instead a 27 % fall is observed, whereas the number of germ cells doubles in controls. After 17 days, the rate of germ cell degeneration is similar in AMH-treated and in control ovaries, 13·2 % germ cells are lost from control and 13·8 % from AMH-treated ovaries per day in culture. These data indicate that AMH acts essentially by inhibiting germ cell proliferation and not by accelerating their degeneration.

The effect of increasing doses of AMH upon number and meiotic maturation of germ cells in 14·5-day-old fetal rat ovaries cultured 5 days is shown in Fig. 2, by comparison with control cultures. AMH, at a concentration of 3 $\mu g\,ml^{-1}$, decreases germ cell number by 82 %, down to a threshold concentration of approximately 1 $\mu g\,ml^{-1}$. Meiotic maturation is affected only at the highest AMH dose employed. The percentage of oogonia and of cells in the initial stage of meiotic prophase is increased, but this is due essentially to the selective loss of germ cells in advanced stages of meiotic maturation: the absolute number of oogonia and leptotene germ cells is similar in AMH-treated and control cultures.

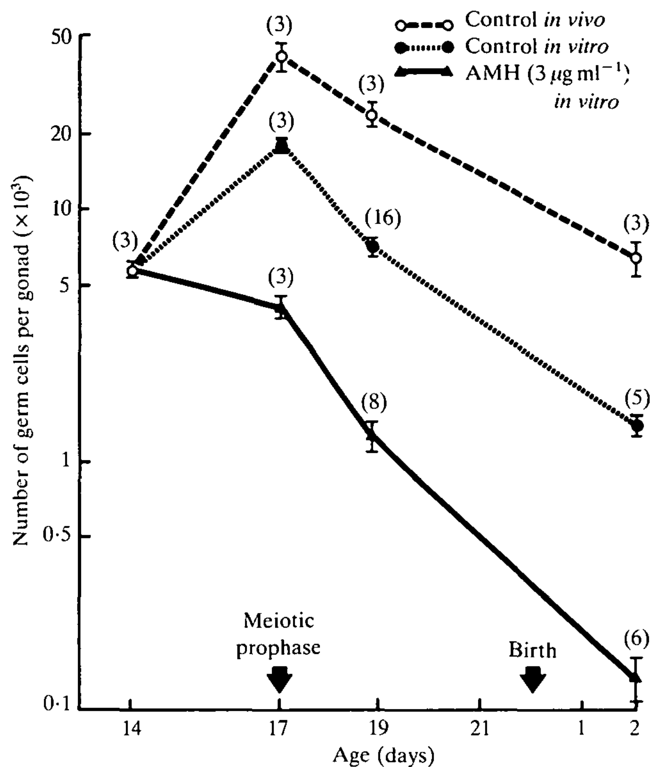

Fig. 1. Number of germ cells in developing rat ovaries, *in vivo* and *in vitro*. *In vivo*, the number of germ cells increases markedly between 14 and 17 days, and then decreases when meiotic maturation is initiated. Control cultured ovaries show roughly the same pattern, but the number of germ cells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

AMH effect is modulated by the length of the culture period and by the age of the ovaries at explantation: AMH retains its effect upon germ cell number, but not meiotic maturation, in cultures initiated at 14 days and terminated after 3 days (Fig. 3A). Both effects are enhanced in 14-day-old ovaries cultured 10 days: oogonia and leptotene germ cells are still present in AMH-treated ovaries, and not in control ones (Fig. 3B). AMH has no effect upon ovaries explanted at 20 days *p.c.* (Fig. 3C), or

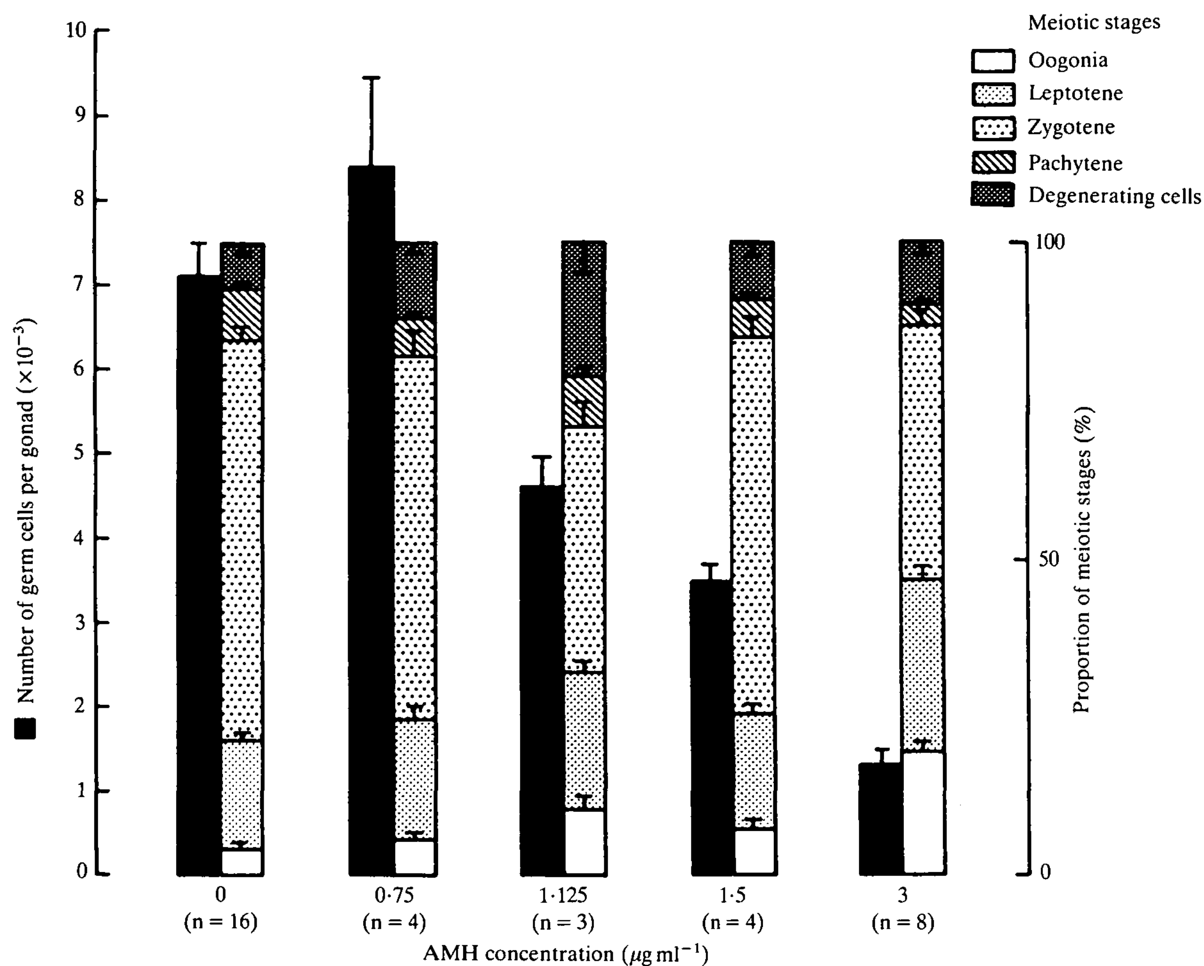

Fig. 2. Effect of increasing concentrations of purified bovine AMH upon number and meiotic maturation of germ cells in 14-day-old fetal rat ovaries cultured 5 days *p.c.* AMH reduces the total number of germ cells per fetal ovary in a dose-dependent manner. The threshold concentration of AMH is between 0·75 and 1·125 μg ml^{-1}. In contrast, the progression of meiotic prophase maturation is affected only at the highest dose used (see text). Meiotic maturation in control cultured ovaries corresponds to that observed *in vivo* a day earlier (Rivelis *et al.* 1976).

3 days *postpartum* (Fig. 9A) or upon male germ cells (Fig. 9B): the total number of germ cells in 14-day-old fetal testes cultured 5 days is 11·445 ± 2·043 in controls, and 14·023 ± 2·578 in cultures exposed to 3 μg ml^{-1} AMH.

Gonadal volume

AMH significantly reduces gonadal volume. In 14-day-old fetal ovaries cultured 5 days, mean gonadal volume ± S.E.M. is 0·10157 ± 0·0044 mm^3 in controls and 0·0375 ± 0·0073 in ovaries exposed to 3 μg ml^{-1} 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 μg ml^{-1} or over (Fig. 4), differentiating cord-like structures are irregularly disseminated in the gonadal blastema. Inside these structures, somatic cells assume an epithelioid appearance and become polarized, with nuclei aligned against a discontinuous basement membrane, often lined at the exterior by small fusiform mesenchymal cells. At the surface of the gonad, a layer of flattened connective cells resembles a differentiating tunica albuginea. Ultrastructural examination indicates that groups of epithelial cells develop in the gonadal blastema (Fig. 5B), forming cord-like structures resembling differentiating seminiferous tubules (Fig. 6). These epithelial cells, joined by typical interdigitations, acquire a globular nucleus and an enlarged cytoplasm, less electron-dense than that of other cell types. The amount of rough endoplasmic reticulum does not increase.

Control cultures, performed in the absence of AMH, are characterized by a homogeneous blastema, containing numerous germ cells (Fig. 4C). These are located in nests separated by somatic cells with a sparse, electron-dense cytoplasm (Fig. 5A).

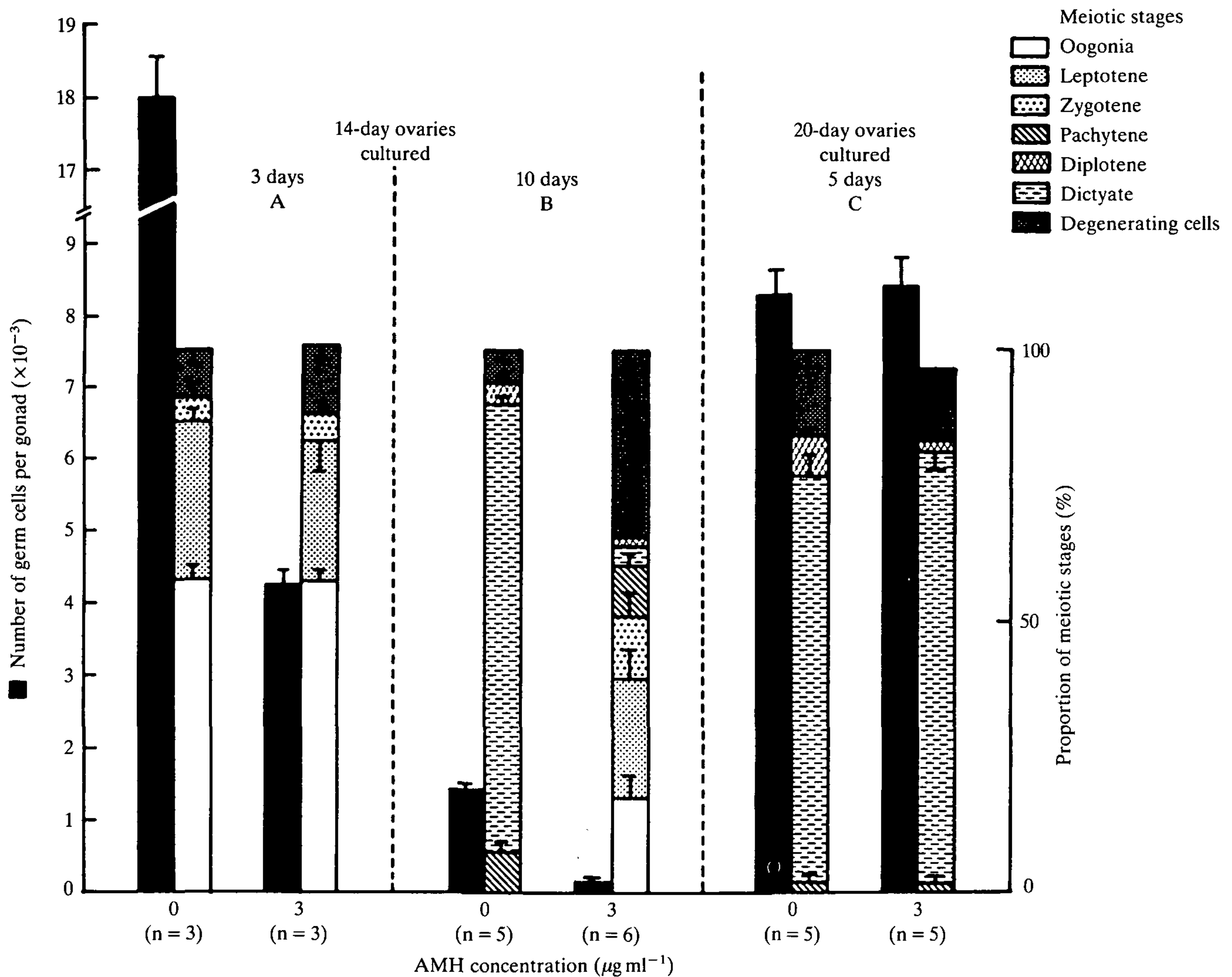

Fig. 3. Effect of the culture period upon the response of fetal rat ovaries to bovine AMH. (A) 14-day-old ovaries cultured 3 days: in AMH-treated cultures, the number of germ cells is reduced by 77 %. Progression of meiotic maturation is not significantly affected. (B) 14-day-old ovaries, cultured 10 days. In AMH-treated cultures, germ cell number is reduced by 89 %, cell degeneration is increased, and meiotic maturation is retarded. (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. Meiotic maturation in control cultured ovaries corresponds to that observed *in vivo* a day earlier (Rivelis *et al.* 1976).

AMH-induced structural modifications are enhanced in cultures maintained 10 days (Fig. 7). Cord-like structures are more frequent and conspicuous. They can be distinguished from authentic seminiferous tubules of 13- or 14-day-old testes only by the fact that they contain few germ cells, some of which may have entered meiotic prophase (Fig. 7B). Polarization of epithelial cells along a basement membrane is illustrated on Fig. 7B and C. This basement membrane contains dense deposits of fibronectin and laminin (Fig. 8). In control ovaries (Fig. 7D), ovarian follicles have not yet developed. Germ cells, in the diplotene or dichtyate stage, are disseminated in the gonadal blastema. Immunohistochemical staining reveals a tendency towards compartmentalization of the blastema by laminin deposits, but these are relatively faint and discontinuous, and do not surround discrete cords, as in the AMH-treated ovaries. In contrast to these, control ovaries contain little or no fibronectin.

AMH induces no structural modifications in cultures initiated at 20 days *p.c.* and later, and does not interfere with folliculogenesis. In control and AMH-treated cultures initiated at 20 days *p.c.* and maintained 5 days, some ovocytes are surrounded by a circle of somatic cells. Formation of ovarian follicles is more advanced in ovaries explanted at 3 days

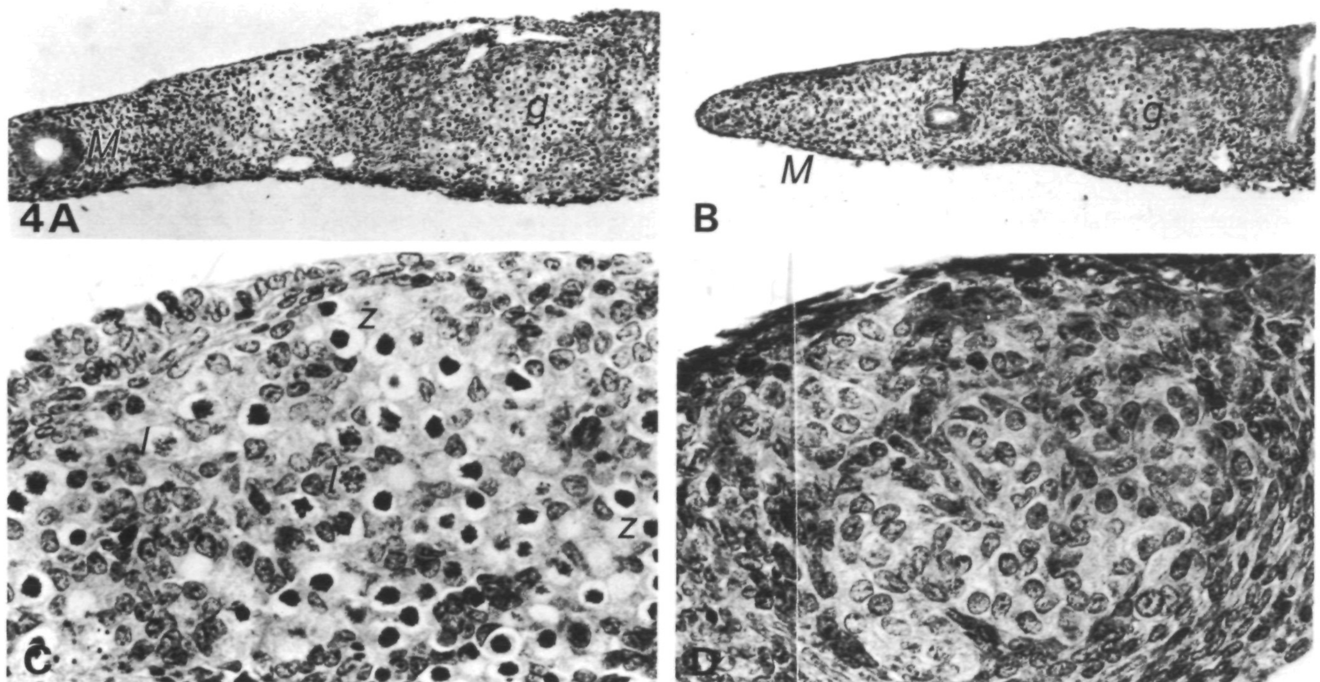

Fig. 4. Effect of AMH ($3\,\mu g\,ml^{-1}$) upon histological structure of 14-day-old ovaries, cultured 5 days. (A,C) Controls; (B,D) treated with AMH. (A) Fetal rat ovary and Müllerian duct cultured in control medium. The Müllerian duct (M) is maintained, and the gonad (*g*) is well developed. ×105. (B) Same, cultured in the presence of $3\,\mu g\,ml^{-1}$ bovine AMH. The Müllerian duct is no longer visible and gonadal volume is reduced. The arrow indicates a mesonephric tubule. *g*, gonad; *M*, regressed Müllerian duct. ×105. (C) High-power view of fetal rat ovary in control medium. Note numerous germ cells, mostly in zygotene (*z*) phase of meiotic prophase. *l*, leptotene. ×425. (D) Same, cultured in the presence of $3\,\mu g\,ml^{-1}$ AMH. Developing seminiferous cord-like structures, containing epithelial-like cells, but few germ cells, are detectable on this section. ×425. All sections are stained by haematoxylin–eosin.

postpartum in the presence or absence of AMH (Fig. 9B).

Tests for endogenous AMH production

Within the limits of sensitivity of our immunohistochemical method, no AMH immunoreactivity was induced in fetal ovaries by AMH treatment. In fetal testes studied *in vivo*, a positive reaction was seen in the cytoplasm of developing Sertoli cells in only one out of the three animals studied at 13 days + 18 h, and in all the four animals studied at 14 days + 8 h (Tran, Picard, Campargue & Josso, 1987).

Müllerian ducts

Müllerian regression, witnessed by narrowing of the lumen, epithelial disorganization and formation of a ring of connective tissue around the epithelium, occurs in a dose-dependent manner in all cultures exposed to a 0·75 to $1{\cdot}125\,\mu g\,ml^{-1}$ concentration of AMH at 14 days. At AMH concentrations $1{\cdot}5\,\mu g\,ml^{-1}$ or over, the Müllerian duct has totally disappeared (Fig. 4B). In all control cultures, Müllerian ducts are normal (Fig. 4A).

Discussion

The *in vitro* model designed to investigate the effect of bAMH upon the fetal ovary is reliable, since the results obtained in control cultures are comparable to those reported previously in the same species, *in vitro* by Rivelis, Prépin, Vigier & Jost (1976) and Prépin *et al.* (1985*a,b*) and *in vivo* by Beaumont & Mandl (1962) and Bézard & 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 germ cells enter the meiotic prophase and many fall victims to waves of degeneration. *In vitro*, the evolution of meiotic maturation is identical to that observed *in vivo*, with approximately a 24 h lag (Rivelis *et al.* 1976). Our results show that AMH interferes essentially with oogonial replication, whereas, after entry in meiotic prophase, the rate of germ cell loss is similar in control and AMH-treated cultures (Fig. 1). Prépin *et al.* (1985*a*) also found that the decrease in ovarian germ cell population elicited by coculture with fetal testicular tissue was due to inhibition of oogonial replication. In contrast, AMH does not prevent germ cells from entering meiosis,

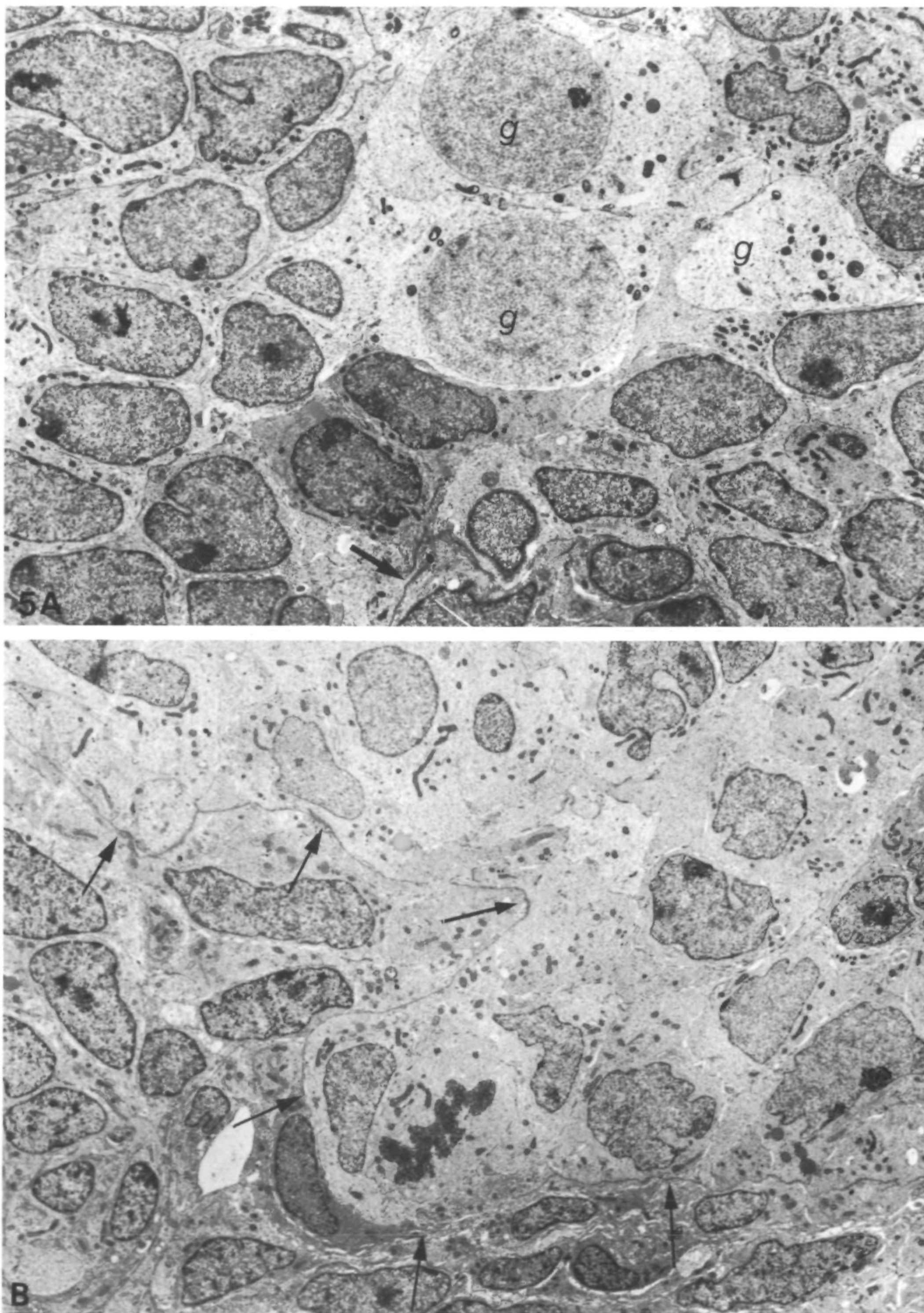

Fig. 5. Differentiation of epithelial cells in 14-day-old fetal rat ovaries cultured 5 days. ×2850. (A) In controls, somatic cells are characterized by a sparse, electron-dense cytoplasm. In some places (arrows), a condensation of the extracellular matrix, resembling a basement membrane is visible. These structures, however, are few and poorly developed. Germ cells (*g*) have a large, round nucleus. (B) In the presence of AMH ($3\,\mu g\,ml^{-1}$) groups of somatic epithelial cells, limited by a developing basement membrane (arrows), are characterized by uniformly granular nuclei with irregular nuclear boundaries and by an abundant, clear cytoplasm. These cells resemble differentiating Sertoli cells. Between these islands of epithelial cells, other somatic elements are differentiating into mesenchymal cells.

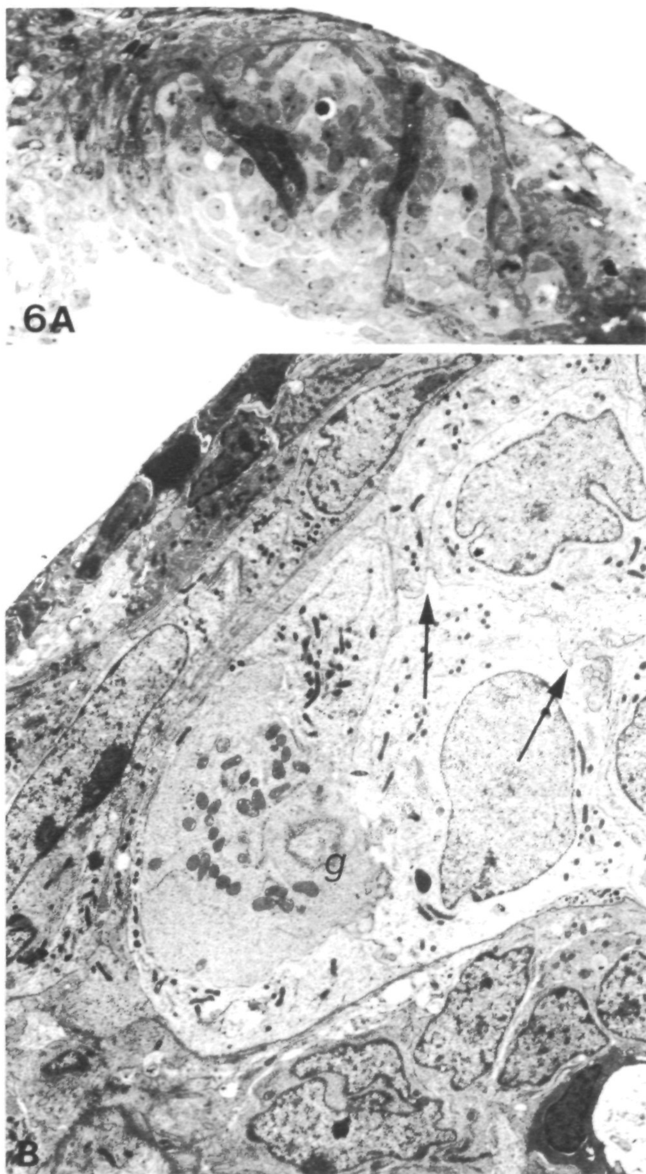

Fig. 6. Seminiferous cord-like structures in the anterior pole of a 14-day-old fetal rat ovary, cultured 5 days in the presence of AMH ($3\,\mu g\,ml^{-1}$). (A) Semithin section, showing seminiferous cord-like structures, separated by dense fusiform mesenchymal cells. ×285. (B) Electron micrograph showing somatic 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. ×3115. *g*, germ cell.

except at a $3\,\mu g\,ml^{-1}$ concentration, after 10 days in culture. In these conditions, some oogonia are still present and few germ cells have reached late stages of meiotic prophase (Fig. 3). The similarity of our culture model to the freemartin situation is striking. At the time AMH secretion is initiated by the bovine testis, germ cells in the prospective ovary of a female united by placental anastomoses to a male twin no longer multiply. Some germ cells fail to enter meiosis and none develop beyond the zygotene or at best pachytene stage (Jost *et al.* 1975; Prépin, Vigier & Jost, 1979). Early interruption of the placental anastomoses protects Müllerian ducts and prospective ovaries from further damage (Vigier *et al.* 1976).

In addition to its inhibiting effect upon the proliferation of oogonia, AMH induces the formation of seminiferous cord-like structures in ovaries exposed to it in culture. AMH-treated ovaries develop structures lined by epithelial cells, delineated by a basement membrane containing laminin and fibronectin. Fibronectin appears very early around differentiating seminiferous tubules and may play a role in the polarization of Sertoli cells (Paranko, Pelliniemi, Vaheri, Foidart & Lakkala-Paranko, 1983). Epithelial cells contained in these structures exhibit some characteristics of developing Sertoli cells, namely globular nuclei, clear, abundant cytoplasm and interdigitations (Magre & Jost, 1980). Their rough endoplasmic reticulum is, however, not enlarged and this may perhaps explain why we have been unable to detect the presence in these cells of immunoreactive AMH, which is localized in this region of the fetal Sertoli cell (Tran *et al.* 1982; Hayashi *et al.* 1984). However, AMH could not usually be detected either 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. Seminiferous tubules containing immunoreactive AMH (Vigier, Tran, Legeai, Bézard & Josso, 1984*b*) develop in approximately half freemartin gonads, but only in the late part of gestation (Jost *et al.* 1973, 1975).

A large body of literature deals with efforts to reproduce the freemartin model experimentally by transplanting, grafting or culturing fetal ovaries. Conflicting results have been reported. For some authors, transplantation of fetal ovaries under the kidney capsule of adult males, but not females, is sufficient to induce masculinization (Buyse, 1935; Taketo, Merchant-Larios & Koide, 1984) but Turner (1969) observes formation of seminiferous tubules only in fetal ovaries transplanted into testes, but not kidneys, of adult male rats. In general, fetal testes have been found to be more effective ovarian modifiers than adult ones (Holyoke, 1957; Beber, 1957; Holyoke & Beber, 1958). Using 15-day-old ovaries cotransplanted with a fetal testis, MacIntyre, Baker & Wykoff (1956) report severe growth inhibition, occasional development of seminiferous tubules, some of which contain oocytes. Odżeński, Rogulska, Bałakier, Brzozowska, Rembiszewska & Stepinska

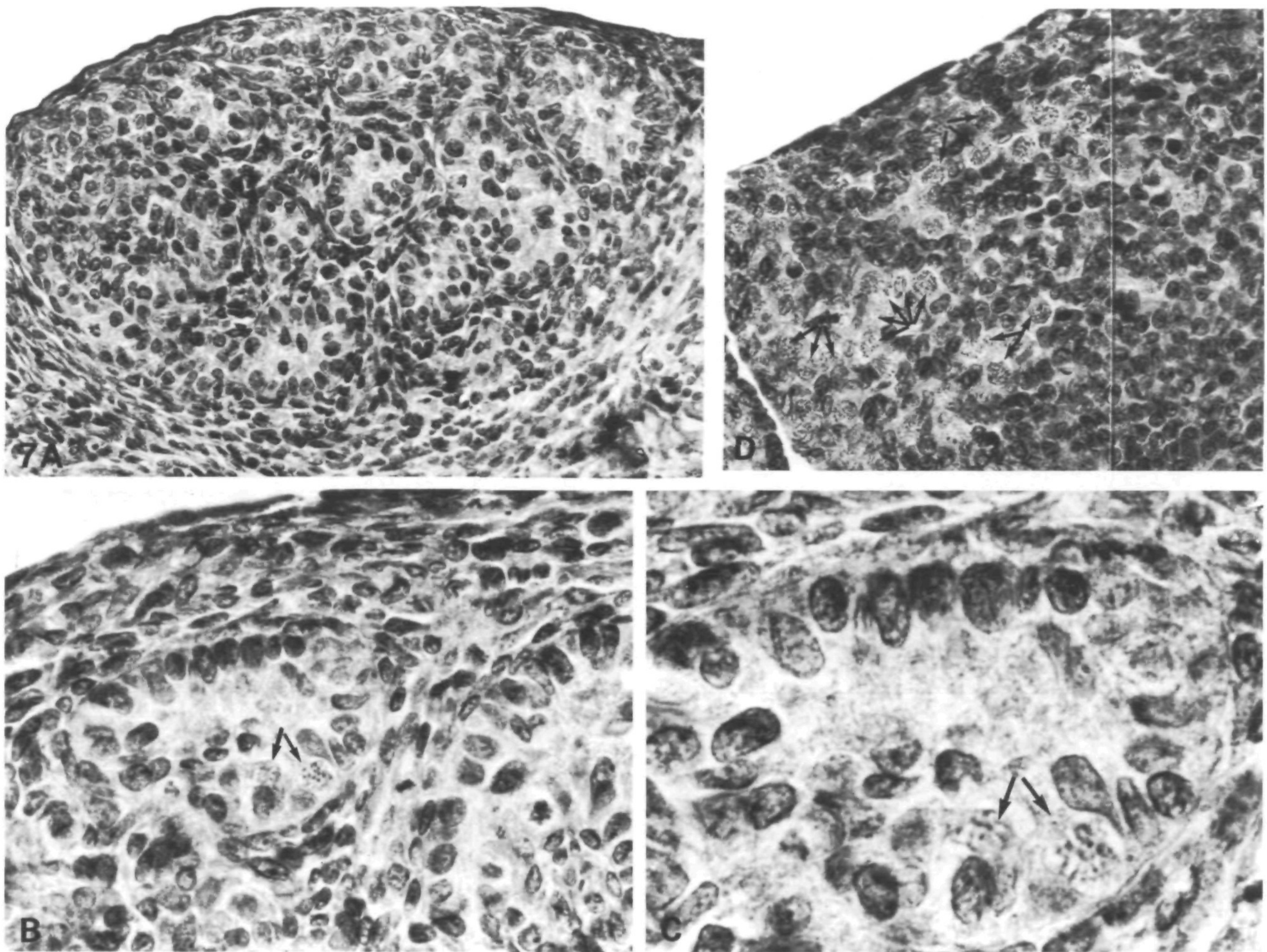

Fig. 7. Effect of AMH upon the structure of 14-day-old fetal rat ovaries cultured 10 days. Haematoxylin–eosin stain. (A,B,C) Ovaries cultured in the presence of $1{\cdot}5\,\mu g\,ml^{-1}$ AMH; (D) Control. (A) Note conspicuous seminiferous cord-like structures, delineated by a basement membrane, lined by small fusiform mesenchymal cells. ×290. (B) Note layer of connective cells, resembling a tunica albuginea, separating a developing seminiferous cord from the surface of the gonad. ×450. (C) High-power view of B: note polarization along the basement membrane, of epithelioid somatic cells, and presence of two germ cells in meiotic prophase (arrows) in the seminiferous cord-like structure. ×1125. (D) Ovary cultured in control medium. Germ cells, usually in pachytene or diplotene stage of meiotic prophase (arrows), are dispersed in an undifferentiated blastema. No follicles are visible. ×390.

(1976) and Mangoushi (1977) observe growth inhibition and germ cell loss in fetal mouse or rat ovaries cotransplanted with fetal testes under the kidney capsule, but present no convincing evidence for seminiferous tubule formation. Prépin *et al.* (1985*a*) show severe germ cell loss but no masculinization in rat fetal ovaries exposed *in vitro* from 13 to 17 days *p.c.* to conditioned medium of fetal or postnatal rat testes. In contrast, Rashedi, Maraud & Stoll (1983) by grafting chick embryonic testes into the extracoelomic cavity of female embryos observe, in some cases, complete gonadal sex reversal accompanied by spermatogenesis. Finally, while Byskov & Saxen (1976), Byskov (1978) and Evans, Robb, Tuckett & Challoner (1982) report that diffusible fetal testicular secretions inhibit the progression of meiosis in the fetal ovary, their results have not been reproduced by others (Stein & Anderson, 1981; Prépin *et al.* 1985*a*). The bewildering variety of ovarian lesions produced by exposure to fetal testes can be explained by differences in the species and protocols used. In this context, the tendency of XX/XY chimaeras to develop into normal males (McLaren, 1984) should be mentioned. Apparently, a critical proportion of XY cells can induce XX cells to participate in the formation of a testis.

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, Christian, Wachtel & Koo (1976) and Wachtel, Hall, Muller & Chaganti (1980) have

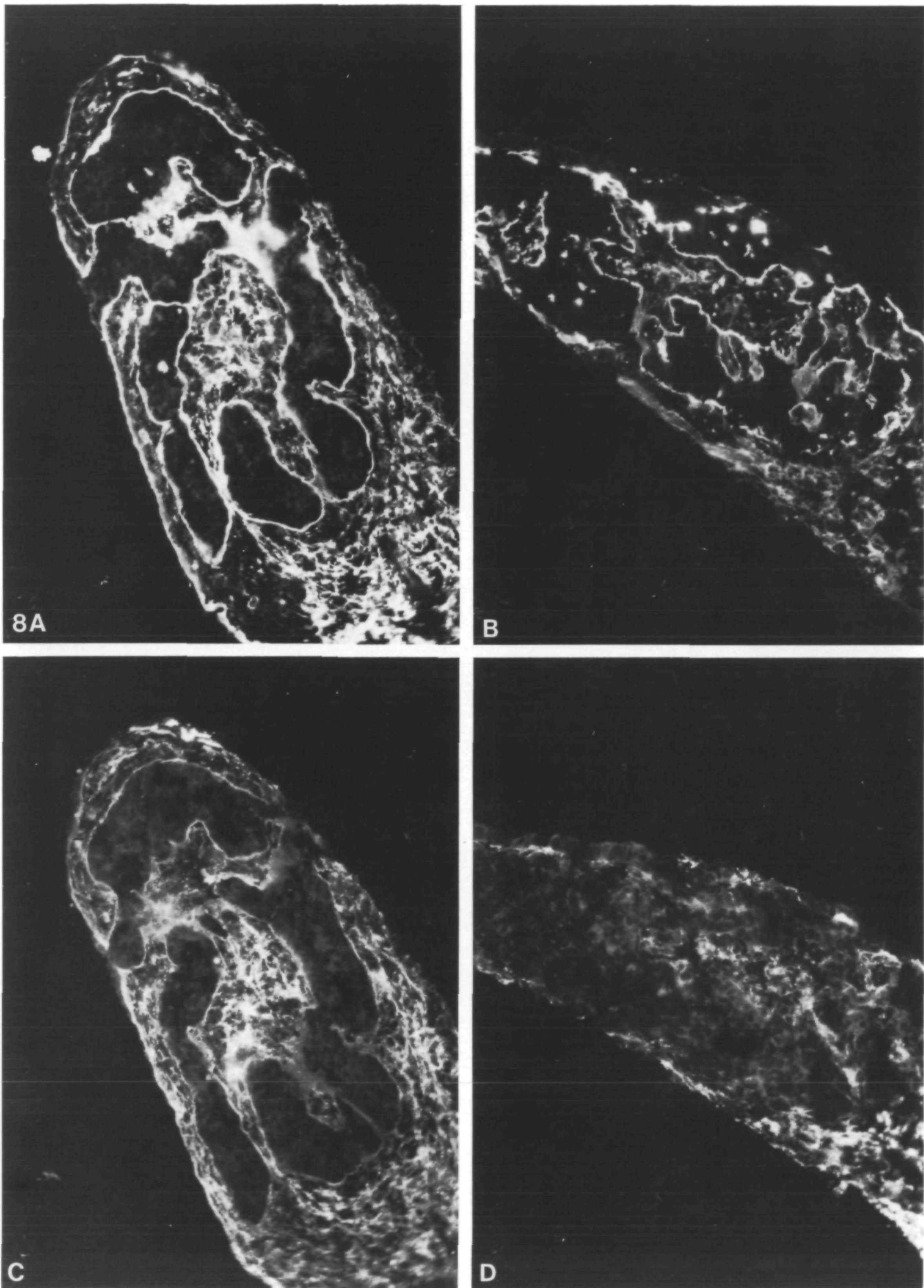

Fig. 8. *Immunohistochemical localization of laminin (A,B) and fibronectin (C,D) in 14-day-old fetal rat ovaries,* cultured 10 days in the presence (A,C) or absence (B,D) of AMH, $3\,\mu g\,ml^{-1}$. Note the heavy deposit of laminin and to a lesser degree fibronectin around the seminiferous cord-like structures induced by AMH treatment. ×300.

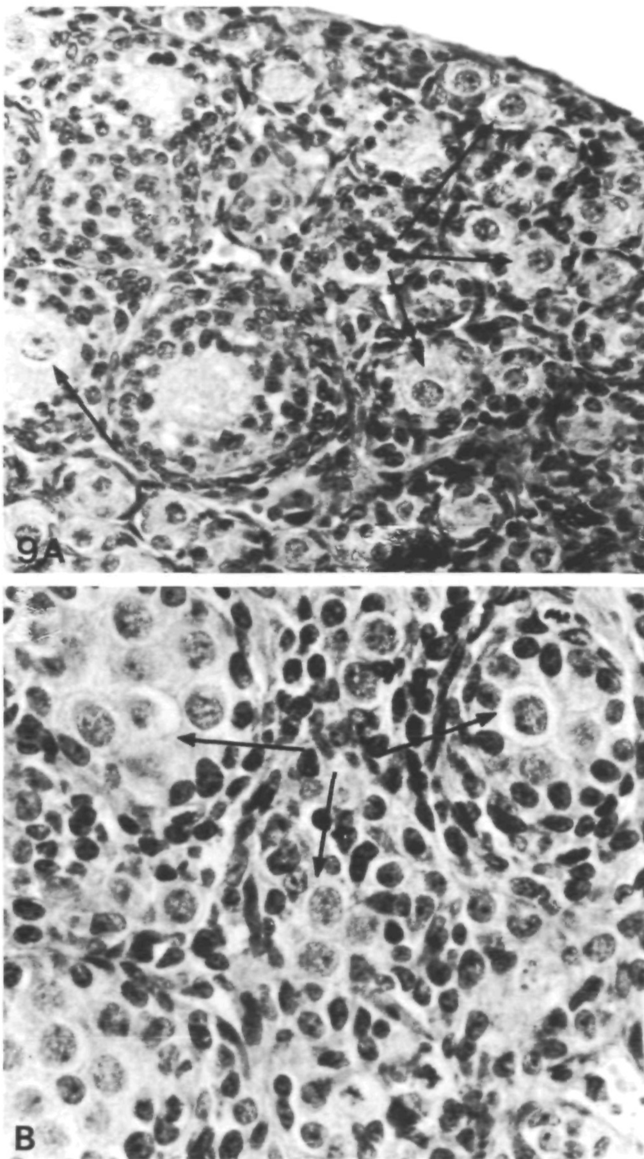

Fig. 9. Lack of effect of AMH, $3\,\mu g\,ml^{-1}$, upon postnatal rat ovaries and fetal testes, cultured 5 days. (A) Rat ovary, aged 3 days *post partum*: normally developing germ cells are contained in developing follicles (arrows). Haematoxylin–eosin. ×360. (B) 14-day-old fetal rat testis: numerous germ cells are visible (arrows), both inside and outside seminiferous tubules. Haematoxylin–eosin. ×585.

suggested that H-Y antigen is the culprit, but no conclusive evidence has been obtained in favour of the testis-inducing role of this gene product (McLaren, Simpson, Tomonari, Chandler & Hogg, 1984). Other investigators (Jost *et al.* 1972; Vigier, Prépin, Perchellet & Jost, 1977; Stoll, Rashedi & Maraud, 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 & Josso, 1984), we are now in a position to confirm this hypothesis. In our hands, AMH added to culture medium consistently reduces germ cell number and induces 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 formation of testicular cords (Merchant, 1975; Merchant-Larios & Centeno, 1981). Furthermore, some of these cord-like structures contain a few germ cells (Fig. 7D). AMH has no effect upon fetal ovaries at day 20 and does not perturb formation of ovarian follicles, indicating that a critical phase of sensitivity to AMH, previously demonstrated for Müllerian ducts (Picon, 1969; Taguchi, Cunha, Lawrence & Robboy, 1984) can also be delineated for germ cells. In the ovary, this critical phase is terminated at the time of differentiation of granulosa cells, the cells which produce AMH in the ovary (Vigier, Picard, Tran, Legeai & Josso, 1984*a*). Bovine AMH has no effect upon male germ cells, proving its effect upon young fetal ovaries is not due to nonspecific toxicity. A role for AMH in the demise of XX germ cells placed in a testicular environment is less clear. Whereas germ cells apparently thrive in sex-reversed gonads of genetic female birds (Rashedi *et al.* 1983), most mammalian testicular XX germ cells degenerate during the neonatal period (McLaren, 1981). If AMH was involved, one would expect an earlier effect.

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 may be required at some critical point after triggering of testicular organogenesis through some other Y-directed stimulus, to regulate germ cell proliferation, to complete Sertoli cell differentiation and to assist seminiferous cord edification. We have recently shown that passive immunization of male rabbit fetuses against AMH, leading to persistence of their Müllerian derivatives, does not impair testicular development or lead to meiotic entry of germ cells immediately after birth (Picard, Tran, Vigier & Josso, 1983; Tran, Picard, Vigier, Berger & Josso, 1986). However, this experiment does not totally rule out a role for AMH in testicular development, since the timing of the immunization, geared to affect Müllerian development,

may have been inadequate for the demonstration of other physiological effects. Exciting developments of research in this field await us in the near future.

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