

Extracellular Matrix Regulates Sertoli Cell Differentiation, Testicular Cord Formation, and Germ Cell Development In Vitro

MARK A. HADLEY,* STEPHEN W. BYERS,* CARLOS A. SUÁREZ-QUIAN,[‡]
HYNDA K. KLEINMAN,[§] and MARTIN DYM*

**Department of Anatomy and Cell Biology, School of Medicine–School of Dentistry, Georgetown University, Washington DC 20007; National Institutes of Health, [‡]National Institute of Child Health and Human Development, and [§]National Institute of Dental Research, Bethesda, Maryland 20205*

ABSTRACT Sertoli cell preparations isolated from 10-day-old rats were cultured on three different substrates: plastic, a matrix deposited by co-culture of Sertoli and peritubular myoid cells, and a reconstituted basement membrane gel from the EHS tumor. When grown on plastic, Sertoli cells formed a squamous monolayer that did not retain contaminating germ cells. Grown on the matrix deposited by Sertoli-myoid cell co-cultures, Sertoli cells were more cuboidal and supported some germ cells but did not allow them to differentiate. After 3 wk however, the Sertoli cells flattened to resemble those grown on plastic. In contrast, the Sertoli cells grown on top of the reconstituted basement membrane formed polarized monolayers virtually identical to Sertoli cells in vivo. They were columnar with an elaborate cytoskeleton. In addition, they had characteristic basally located tight junctions and maintained germ cells for at least 5 wk in the basal aspect of the monolayer. However, germ cells did not differentiate. Total protein, androgen binding protein, transferrin, and type I collagen secretion were markedly greater when Sertoli cells were grown on the extracellular matrices than when they were grown on plastic.

When Sertoli cells were cultured within rather than on top of reconstituted basement membrane gels they reorganized into cords. After one week, tight junctional complexes formed between adjacent Sertoli cells, functionally compartmentalizing the cords into central (adluminal) and peripheral (basal) compartments. Germ cells within the cords continued to differentiate.

Thus, Sertoli cells cultured on top of extracellular matrix components assume a phenotype and morphology more characteristic of the in vivo, differentiated cells. Growing Sertoli cells within reconstituted basement membrane gels induces a morphogenesis of the cells into cords, which closely resemble the organ from which the cells were dissociated and which provide an environment permissive for germ cell differentiation.

In the adult testis the Sertoli cell is a tall, narrow, columnar cell that extends from the basement membrane to the lumen of the seminiferous tubule. Sertoli cells have a basally located, irregularly shaped nucleus and a prominent, supranuclear stalk of cytoplasm. Cytoplasmic branches extend laterally from this main stalk to surround and support the differentiating germ cells (4). Tight junctional complexes between Sertoli cells are formed only in their basal region and func-

tionally divide the seminiferous tubule epithelium into a basal compartment that contains early germ cells and an adluminal compartment that contains more advanced germ cells (2). Substances from the blood that reach the base of the seminiferous tubule have access to the basal compartment. However, since the extracellular route from the interstitium is barred by tight junctions between Sertoli cells, many substances would have to pass through the Sertoli cell cytoplasm to reach germ

cells in the adluminal compartment. Thus, a blood–testis permeability barrier exists. By compartmentation of the seminiferous tubule epithelium and subsequent creation of a specific adluminal microenvironment, Sertoli cells are considered to regulate germ cell development.

In an effort to determine the role of the Sertoli cell within the complex seminiferous tubule epithelium, Sertoli cells have been enzymatically dissociated from the testis and cultured (1, 25, 31). However, their squamous appearance in conventional culture bears little resemblance to the highly polarized Sertoli cell observed *in vivo*, and germ cells associated with these cultures fail to differentiate. Germ cell differentiation *in vitro* has generally only been obtained in organ culture of testis (19, 24), although Gerton and Millette (6) have shown generation of flagella by isolated mouse round spermatids in culture.

There now exists a great deal of evidence that extracellular matrix can profoundly influence cell growth, metabolism, and differentiation *in vitro* in a number of cell types (for reviews see references 7, 11, 14, and 20). Recently, several laboratories, including our own, have had some success in improving the Sertoli cell culture system by modifying the substratum on which the cells are grown (18, 26, 28). These modifications have included growing Sertoli cells on individual matrix components, type I collagen gels, and extracellular matrix extracts of the testis. In general, these experiments have suggested that extracellular matrix can modulate the morphology and function of Sertoli cells in culture.

In this article we demonstrate a better maintenance of Sertoli cell phenotype in cultures on two extracellular matrix preparations than in cells grown on plastic. Both matrix substrates—a detergent-extracted matrix from a Sertoli-myoid cell co-culture and a reconstituted basement membrane (RBM)¹ gel from the EHS tumor (15)—enhance Sertoli cell morphology and protein secretion, but the RBM maintains a more highly polarized and differentiated Sertoli cell phenotype. In addition, when Sertoli cells are cultured within a RBM gel they reorganize into cords that closely resemble the original seminiferous cords from which they were dissociated. Germ cells within the *in vitro* cords continue to develop.

MATERIALS AND METHODS

Culture Media: Dissociation medium (DM) consisting of Dulbecco's minimum essential medium (Gibco Laboratories Inc., Grand Island, NY) supplemented with 100 U/ml penicillin, 0.25 μ g/ml fungizone, 100 μ g/ml streptomycin (Gibco Laboratories Inc.) was used throughout the isolation procedure. Serum-free defined medium (SFDM) consisting of the DM plus 100 ng/ml follicle-stimulating hormone (NIADDK-oFSH-16), 2 μ g/ml insulin, 5 μ g/ml human transferrin, 50 ng/ml vitamin A, 200 ng/ml vitamin E, 10^{-9} M hydrocortisone, 10 ng/ml epidermal growth factor, 10^{-8} M testosterone, 10^{-8} M estradiol, 2 mM glutamine, 5 ng/ml sodium selenate, 1 mM sodium pyruvate, 22 mM sodium lactate, with or without 3 mg/ml cytosine arabinoside (all from Sigma Chemical Co., St. Louis, MO) was used in the culture of Sertoli cells. In addition, cultures were supplemented with 50 ng/ml of vitamin C (Sigma Chemical Co.) daily.

Sertoli Cell Preparation: Sertoli cells were isolated using a modification of the method previously reported by Rich et al. (21). Testes from 10-day-old Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were removed aseptically, washed in DM, decapsulated, and placed into fresh DM. The tissue was minced into ~3-mm fragments, washed, and

resuspended in DM containing 300 U/ml crude collagenase (CLS II, Worthington Biochemical Corp., Freehold, NJ) and 5 μ g/ml DNase (Type 1, Sigma Chemical Co.) for 10–20 min at 37°C on a shaking water bath (70 cycles/min). The cells were periodically aspirated with a 10-ml pipette. Tubule fragments from this first collagenase digestion were allowed to settle by gravity in a 50-ml centrifuge tube. The supernate was saved to harvest peritubular myoid cells. The sedimented tubules were washed twice with DM, and then a second collagenase digestion was carried out for 10–20 min. Tubule fragments from the second collagenase digestion were harvested by centrifugation, washed twice in DM, and resuspended in DM containing 300 U/ml of a more highly purified collagenase (*Cl. histolyticum*, Gibco Laboratories Inc.) and 5 μ g/ml DNase at 37°C. After 20–30 min with intermittent aspiration, the cell aggregates from this third digestion were harvested by centrifugation, washed twice, resuspended in DM containing 2.5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.), bovine serum albumin (Sigma Chemical Co.), and 5 μ g/ml DNase for 5 min at 37°C and then washed twice again in DM. The Sertoli cell aggregates were then resuspended in SFDM. A sample of the Sertoli cell suspension was further dissociated with trypsin/EDTA to single cells and counted with a hemocytometer. The aggregates were then plated at a density of 4×10^6 cells/cm² in 35-mm cluster⁶ Culture dishes (Costar, Cambridge, MA) in a final volume of 2 ml SFDM. Cells were maintained at 32°C in a humidified incubator in an atmosphere of 95% air and 5% CO₂. The medium was changed twice a week. All steps of this procedure were closely monitored by phase-contrast microscopy. This procedure yields Sertoli cell aggregates of 10–50 cells that contain a 1–5% contamination of germ cells (only up to type B spermatogonia), a 1–2% contamination of myoid cells, and very small (<1%) contamination of Leydig and fibroblastic cells, as revealed by light and electron microscopy.

The supernate from the first collagenase digestion was centrifuged at 60 g. The pellet was discarded and the supernatant was centrifuged at 400 g. This second pellet contained the peritubular myoid cells that were used in co-cultures with Sertoli cells to obtain Sertoli–myoid matrix.

Substrates: Sertoli–myoid matrix was obtained by co-culturing myoid and Sertoli cells at high density for 2 wk and then extracting the cultures with a solution of 1% Nonidet P-40, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, 0.15 M NaCl, 0.005 M EDTA in 0.05 M Tris buffer (pH 7.4). The solution was aspirated vigorously at 4°C until the cells were completely extracted except for an unevenly spread, thin amorphous layer that contained some filaments and nuclei. The dishes were then washed numerous times in sterile H₂O and finally in SFDM before Sertoli cells were plated onto this matrix.

The RBM gel is prepared from an unfractionated high salt/urea extract of the EHS (Engelbreth-Holm-Swarm) tumor which can reconstitute to a porous gel at 35°C (15). Ultrastructurally, this gel is similar in appearance to the lamina densa of basement membranes (15). It has been found to support the growth and maintain a differentiated phenotype of various epithelial cells including hepatocytes and melanocytes. The major components of the gel include laminin, type IV collagen, heparan sulfate proteoglycan, nidogen, and entactin (15). Sertoli cells were either cultured on top of a thin layer (<50 μ m) of RBM (to form monolayers) or within a 1.5-mm thick RBM gel (to form cords).

Morphology: Cell cultures were photographed with an IM-35 Zeiss inverted microscope with phase optics. For brightfield light microscopy and transmission electron microscopy, cells were fixed with 3% glutaraldehyde in 0.2 M *s*-collidine buffer (pH 7.4), postfixated in 1.5% osmium tetroxide and 1% potassium ferrocyanide, dehydrated in ethanol, and embedded in Epon. Semithin (1 μ m) and thin (90 nm) sections were cut on a Sorvall MT-6000 ultramicrotome (DuPont Instruments–Sorvall Biomedical Div., Wilmington, DE) and photographed using a Universal Zeiss microscope with a 63 \times oil planapochromatic objective for light microscopy, and a JEOL 100S electron microscope for electron microscopy. Sections used for electron microscopy were stained with uranyl acetate and lead citrate.

Extracellular Tracer: Lanthanum was used as an electron dense intercellular tracer in some experiments involving *in vitro* cords. The procedure was identical to that used by Dym and Fawcett (2), with the exception that the cultures of *in vitro* cords were immersion, not perfusion, fixed.

Protein Assays: For each substrate, the media from six 35-mm culture dishes were collected over the course of 2 wk for three separate experiments. Cytosine arabinoside was included in all cultures used for protein assays to inhibit the mitosis of any contaminating fibroblasts or myoid cells. Culture media were removed and centrifuged at 1,000 g for 20 min at 4°C. The media were made 2 mM with respect to phenylmethylsulfonyl fluoride and *N*-ethylmaleimide, and the samples were stored at –20°C for no more than 2 wk. Cells were removed from the culture dishes by trypsin/EDTA, dissociated to a single cell suspension, and counted with a hemocytometer.

Total protein was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). SFDM background was subtracted from all samples.

¹ Abbreviations used in this paper: ABP, androgen binding protein; DM, dissociation medium; RBM, reconstituted basement membrane; SFDM, serum-free defined medium.

The data were processed by analysis of variance using Duncan's new multiple range test (32).

Androgen binding protein (ABP) was measured by a radioimmunoassay using an ABP assay kit supplied by the National Institute of Arthritis, Metabolic and Digestive Diseases (9, 10). All samples were measured in one assay. The intra-assay variation was 6%, and the sensitivity was 0.3 ng per tube.

Transferrin was measured using a modification of the radioimmunoassay technique described by Skinner and Griswold (22). In brief, rabbit anti-rat transferrin (Cappel Laboratories, Cochranville, PA) at a dilution of 1/15,000 (100 μ l) and iodinated rat transferrin (100 μ l, 30,000 cpm) were added to test samples and standards (300 μ l). After an incubation at 4°C for 48 h, 100 μ l goat anti-rabbit immunobeads (Bio-Rad Laboratories) was added, and the incubation was continued for 12 h more. The samples were then centrifuged at 2,000 g for 20 min, and the radioactivity in the pellet was determined in a Beckman Gamma DP5500 counter (Beckman Instruments Inc., Palo Alto, CA). All samples were measured in the same assay. The intra-assay variability was 6%, and the sensitivity was 2 ng per tube. Cross-reactivity of human transferrin with anti-rat transferrin antibody was <0.05%.

Radiolabeling, Electrophoresis, and Fluorography: Cultures were incubated overnight on day 4 with methionine-free SFDM. The medium was replaced on day 5 with [³⁵S]methionine (84 μ Ci/ml; New England Nuclear, Boston, MA), and the labeled medium was collected after 24 h. Protein in the labeled medium was precipitated with 10% trichloroacetic acid and then washed three times in acetone. SDS PAGE of the precipitated proteins was carried out in 6% slab gels according to Laemmli (16). Globular proteins and type I collagen were used as molecular weight standards (Sigma Chemical Co.). The gels were prepared for fluorography by impregnation with Enhance (New England Nuclear) according to the manufacturer's instructions. The dried gels were exposed for 1–2 wk at –70°C to Kodak XRP-6 film which was developed in a Kodak RP X-OMAT processor.

In some cases the labeled proteins were incubated with purified bacterial collagenase (Advance Biofactures, Lynbrook, NY) for 1 h at 37°C before electrophoresis. The reaction was stopped by the addition of an equal volume of twice-concentrated electrophoresis sample buffer, and electrophoresis and fluorography were carried out as described above.

Tritiated Thymidine Labeling: After cells had been cultured in SFDM without cytosine arabinoside for 4 or 6 d, 2.5 μ Ci/ml [³H]thymidine (New England Nuclear) was added to the cultures for 16 h. The coverslips were then rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS. After dehydration, the coverslips were dipped in Kodak NTB-2 emulsion and exposed for 1 wk. The emulsion was then developed in D-19 and the cells were stained with 1% methylene blue. Four coverslips from each experimental group were used, and the labeled cells were counted in 100 different fields, each containing 50–200 cells. Statistics were determined using the Student's *t*-test.

RESULTS

Morphology

PLASTIC SUBSTRATE: Sertoli cell aggregates plated onto plastic attached within 2 h and rapidly spread to form areas of confluent squamous monolayers, ~2 μ m high, as described by others (1, 3, 25, 29, 31) (Fig. 1*a*). The cells have little or no supranuclear cytoplasm, flattened nuclei, and Sertoli–Sertoli tight junctions are only occasionally present and usually located in the apical region. The germ cells (spermatogonia) that contaminated the Sertoli cell aggregates at the time of plating adhere only tenuously to the apical surface of the monolayers and are lost within a week, apparently through phagocytosis or medium changes. Cultures can be maintained for up to 4 wk in this manner.

SERTOLI–MYOID MATRIX: Sertoli cell aggregates grown on Sertoli–myoid matrix respond similarly to those grown on a cell-free seminiferous tubule biomatrix (28) or on type IV collagen and laminin (26). Sertoli cells attach within an hour and spread slowly over the substrate (Fig. 1*b*). Although, the cells appear clumped most of them maintain processes that connect them to the substrate. Peripheral to the clumps, the Sertoli cells form a spreading monolayer that eventually covers the culture dish (Fig. 1*b*). The peripheral Sertoli cells have

a more regular, low cuboidal shape with from 1 to 3 μ m of supranuclear cytoplasm. Individual Sertoli cells in these cultures average from 15 μ m in height in clumps to 5 μ m in peripheral areas. The nuclei of these cells are generally rounded or pyramidal, with their long axis often oriented perpendicular to the substrate (Fig. 1*b*). Tight junctions are occasionally present in these cultures but, like those found in cells cultured on plastic, are usually located in the apical region of the cells. The cytoskeleton is more elaborate than that of cells on plastic, having some microtubules that are oriented in an apical–basal manner. The cytoskeleton of cells in the monolayer region of these cultures is similar to that of Sertoli cells grown on type IV collagen and laminin (26).

Some of the germ cells (spermatogonia) associated with the Sertoli cells at the time of plating appear to be encircled by Sertoli cell cytoplasmic extensions and can be maintained for up to 3 wk. These germ cells, however, do not differentiate even in the absence of cytosine arabinoside and are eventually lost through medium changes or phagocytosis in the first two weeks. By 3 wk of culture, most of the Sertoli cells on Sertoli–myoid matrix are in monolayers and have taken on the squamous appearance of cells grown on plastic. Sertoli cells have been maintained on Sertoli–myoid matrix for up to 6 wk.

THIN-LAYERED RBM SUBSTRATE: Sertoli cell aggregates plated on top of a thin film of RBM (<50 μ m) adhere within 15 min, and with time in culture they reorganize to form monolayer patches of highly polarized, columnar cells, each with a basal attachment to the substrate (Figs. 1*c* and 5*a*). The peripheral Sertoli cells slowly migrate outward, extending the diameter of the patch while maintaining their columnar morphology. Individual Sertoli cells within the patches vary in height from 20 μ m on the periphery of the patch to 60 μ m in the center.

The nuclei of these cells are basally located and irregularly oval or pyramidal, with their long axis mostly oriented perpendicular to the substrate (Figs. 1*c* and 2*c*). Sertoli cells on RBM have an extensive, stalklike, supranuclear cytoplasm that contains slender mitochondria, and numerous microtubules and microfilaments, all oriented parallel to the apical–basal cell axis. The cells have abundant Golgi elements, rough and smooth endoplasmic reticulum, lipid, and membrane-limited dense bodies (Fig. 2).

Sertoli–Sertoli junctions can be found between all of the cells. They are very well developed and consistently located in the basal cytoplasm of the monolayers (Fig. 2*b*). The cytoskeleton is well organized, a cortical mat of filaments lines the basal aspect of the cells (Fig. 2*b*), and a perinuclear zone of filaments surrounds the nucleus.

Germ cells that accompany the original Sertoli cell aggregates are maintained for at least 5 wk as spermatogonia in the basal region of the monolayer, beneath the Sertoli–Sertoli tight junctions (Fig. 2*a*). Germ cells do not differentiate in these monolayer cultures but remain as spermatogonia even in the absence of cytosine arabinoside. Sertoli cells can be maintained in these patchlike monolayers for up to at least 8 wk without loss of this differentiated morphology.

THICK-LAYERED RBM: Sertoli cell aggregates cultured within a thick layer of RBM gel (1.5 mm) rapidly reorganize within 24 h of plating to form cords (Fig. 3*a*). These cords continue to form an anastomosing network throughout the first 3 d in culture. The morphology of the Sertoli cells that

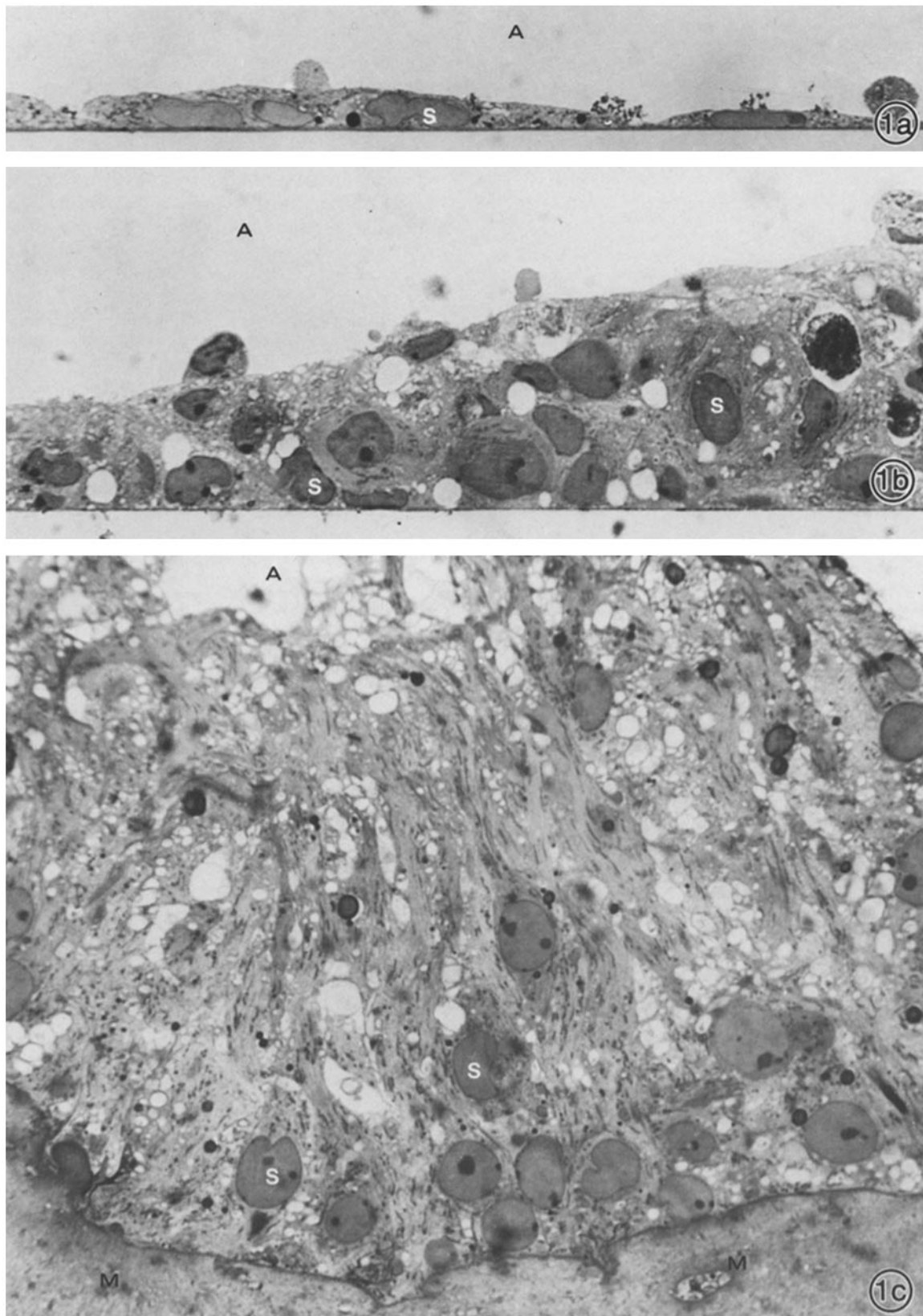


FIGURE 1 Light micrographs of Sertoli cells grown for 2 wk in SFDM. $\times 1,200$. (a) Cells on plastic are very low squamous cells with flattened nuclei (S). (b) On Sertoli-myoid matrix Sertoli cells are still clumped in some areas (at right), but many of the cells have spread to form a low monolayer as seen at the left of the figure. (c) Sertoli cells on RBM (M) form high monolayers with extensive cytoplasmic stalks. Sertoli cell nuclei (S) and apical surfaces (A) are indicated.

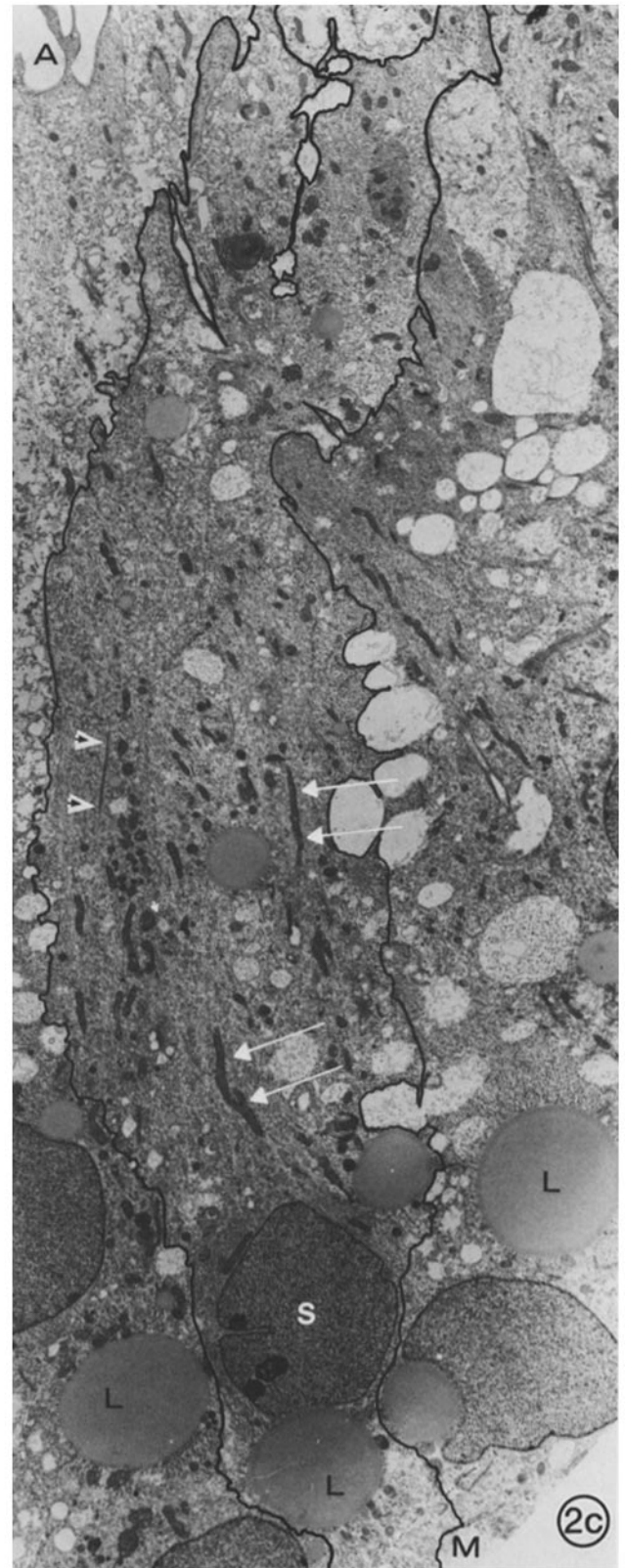
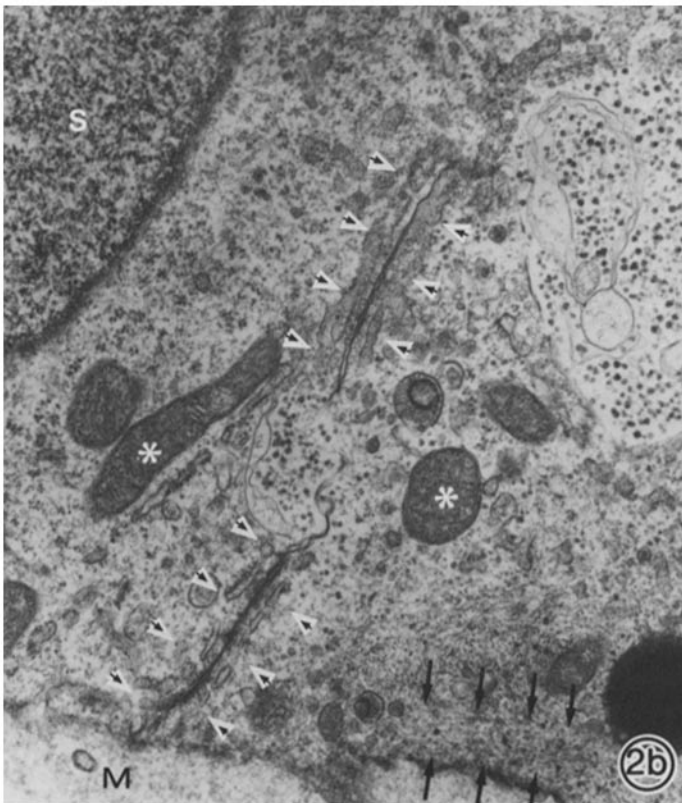
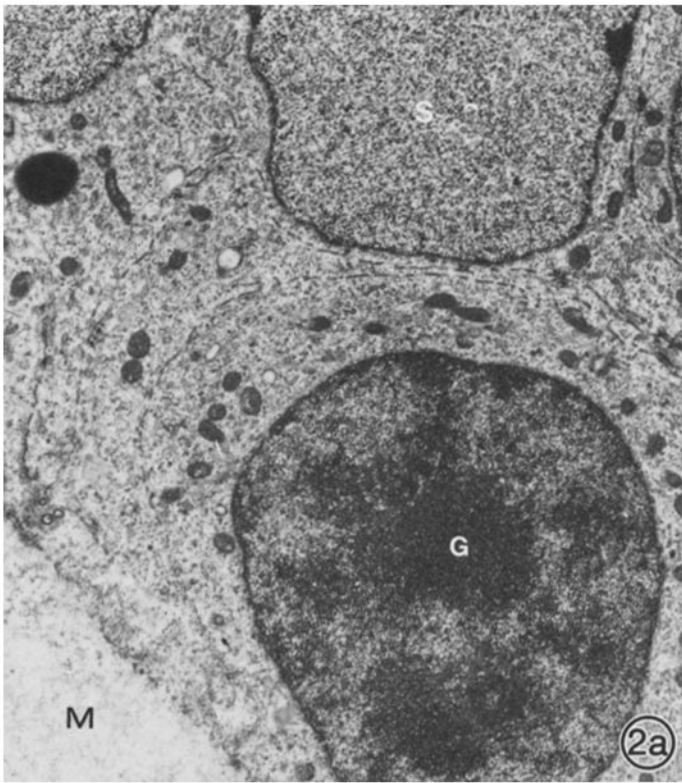
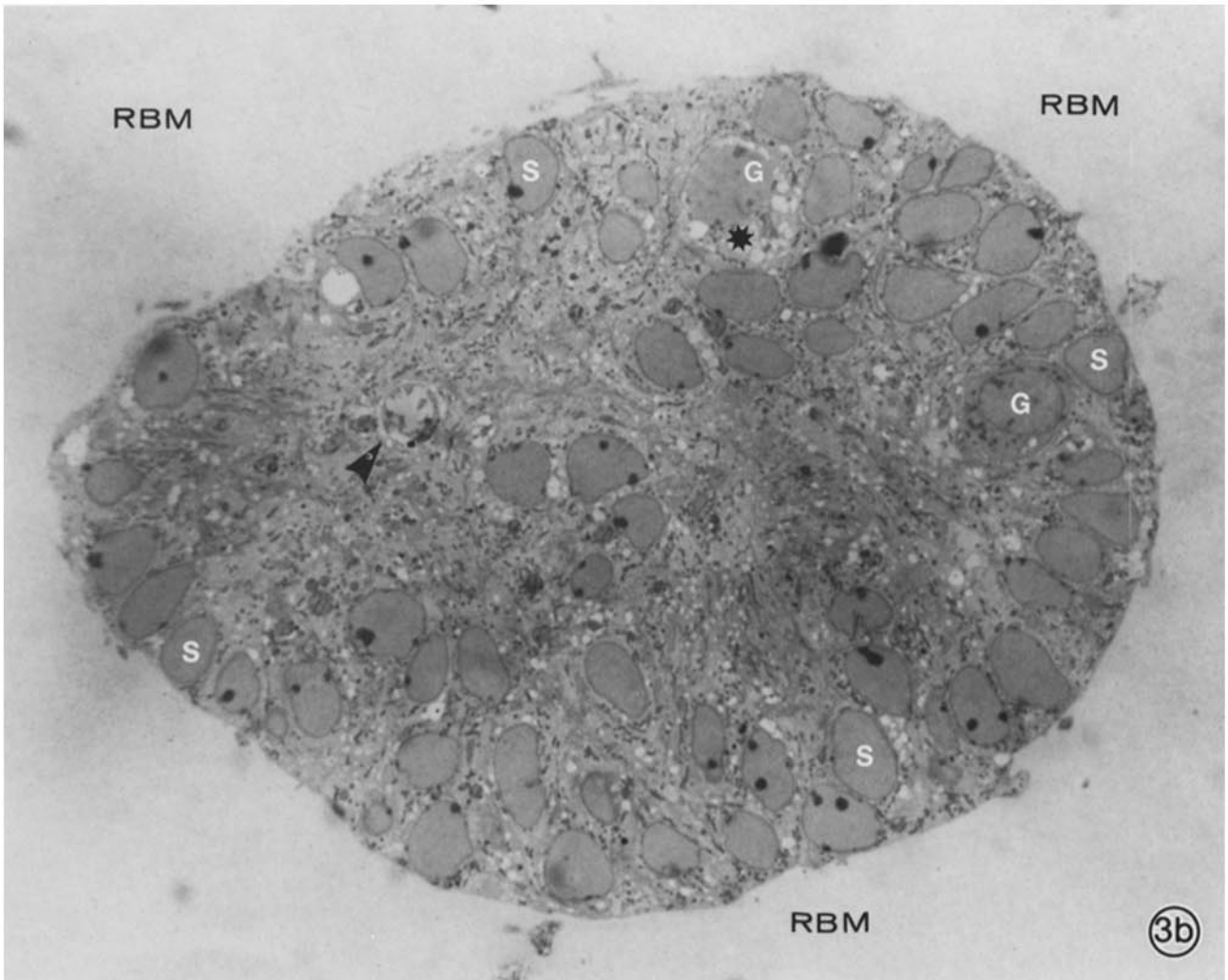
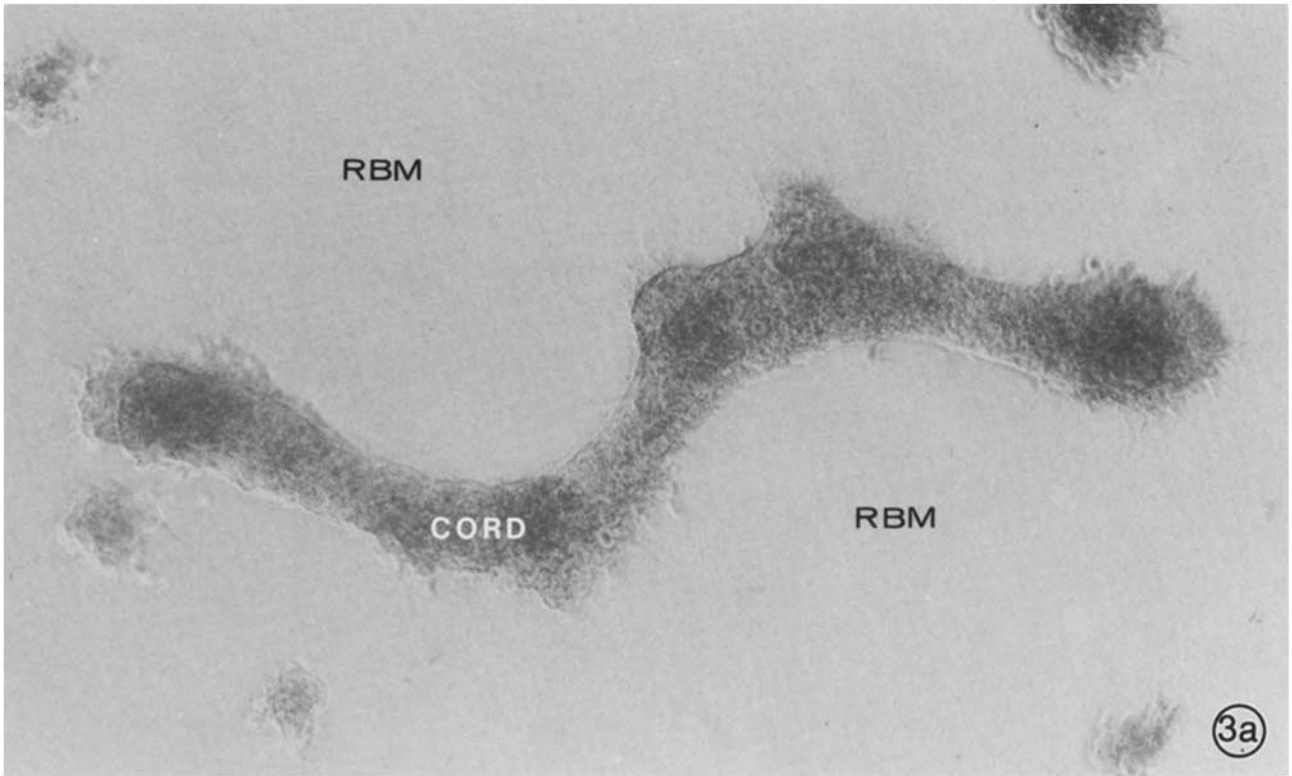


FIGURE 2 Transmission electron micrographs of Sertoli cells grown for 2 wk on RBM in SFDM. (a) This micrograph demonstrates the basal location of a germ cell (spermatogonia) (G) with respect to Sertoli cell nuclei (S) in the cytoplasm of a polarized Sertoli cell monolayer M, RBM. $\times 6,600$. (b) The basal region of a Sertoli cell monolayer. Indicated are the location of a Sertoli-Sertoli tight junctional complex (black-on-white arrowheads), as well as mitochondria (asterisk), Sertoli cell nucleus (S), basal cortical cytoskeleton (black arrows), and RBM (M) $\times 22,000$. (c) This figure shows a tall columnar Sertoli cell with characteristic Sertoli cell nucleus (S), large lipid inclusions (L), and slender rod-shaped mitochondria in the apical cytoplasmic stalk that are oriented parallel to the long axis of the cells (white arrows). A filament bundle can be seen (black-on-white arrowheads). The cell membrane is highlighted in black to demonstrate clearly the extent of one cell. M, the RBM matrix. A, the apical surface. $\times 3,000$.



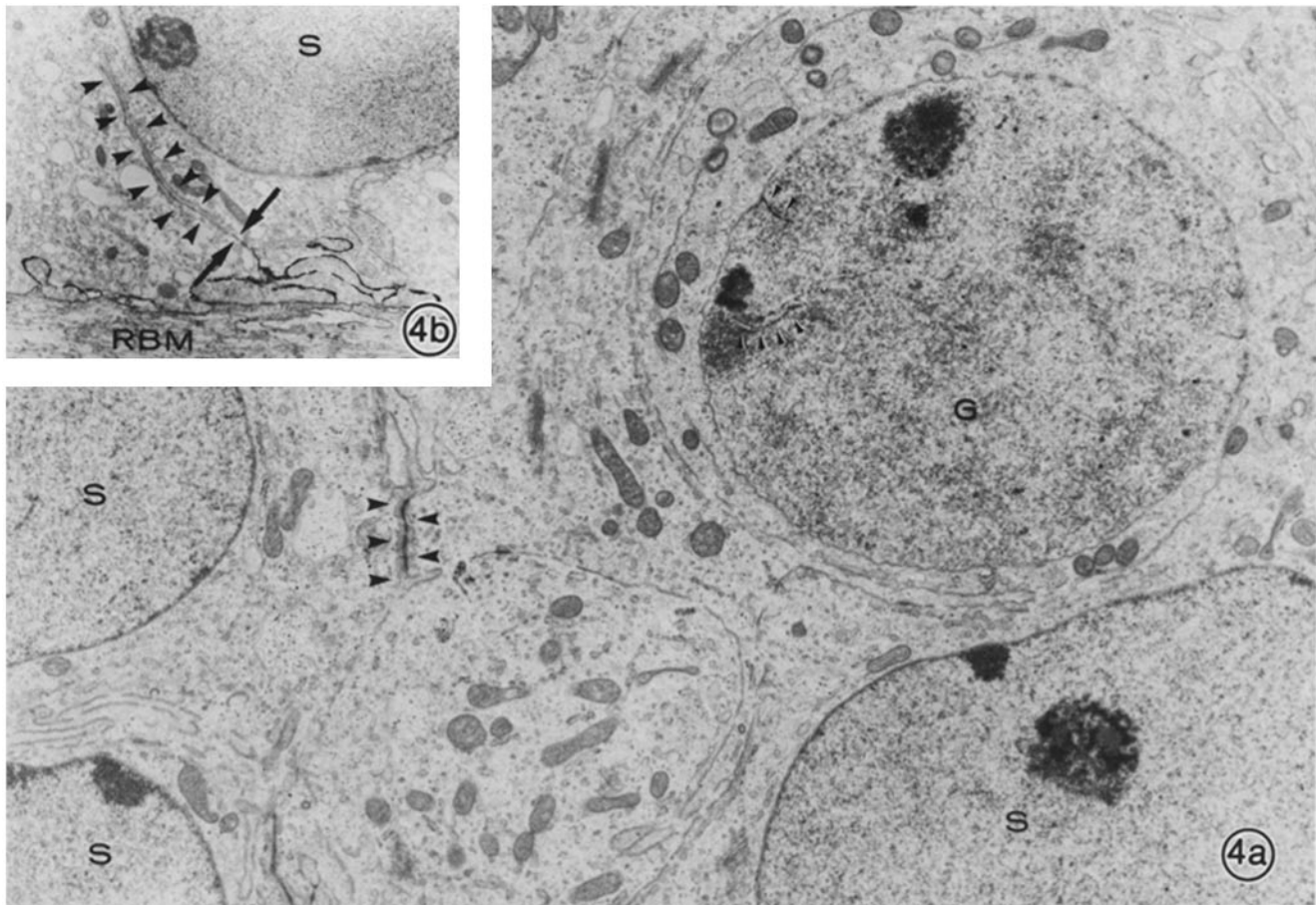


FIGURE 4 (a) This electron micrograph shows a pachytene spermatocyte (C) within an in vitro Sertoli cell cord similar to that in Fig. 3*b*. Synaptonemal complexes are indicated with small black-on-white arrowheads. Sertoli cell nuclei (S) and Sertoli-Sertoli tight junctions (black arrowheads) are shown. Note that the spermatocyte is above the tight junction. $\times 6,000$. (b) This electron micrograph shows the basal region of a cord similar to the cord in a. Lanthanum tracer was added to the fixative and is seen to fill the space between adjacent Sertoli cells up to the tight junctions (the tracer stops at the arrows; the junctions are indicated with arrowheads), but it does not penetrate further. A Sertoli cell nucleus (S) and RBM are also shown. $\times 4,000$.

compose the cords is similar to that of Sertoli cells grown in polarized monolayer on top of RBM, but the apical cytoplasm of the former is not open to the medium but instead projects into the center of the cord, as seen in cross-section (Fig. 3*b*).

In the first week of culture the electron-dense intercellular tracer lanthanum has free access to the intercellular space between the Sertoli cells, including their apical surface in the center of the cords. After a week tight junctions are formed between adjacent Sertoli cells. Once formed, these junctions constitute a permeability barrier that excludes intercellular tracers, such as lanthanum nitrate, from the central compartment of the cords (Fig. 4*b*). The cords are functionally partitioned into peripheral (basal) and central (adluminal) compartments (Fig. 5*b*).

Spermatogonia are peripherally located within the in vitro cords during the first week. By 2 wk in culture spermatogonia still reside in the peripheral compartment, but some germ

cells have differentiated into more advanced cells (up to pachytene spermatocytes) and are found in the central compartment (Figs. 3*b* and 4*a*). Germ cell differentiation occurs only in the absence of the mitotic inhibitor cytosine arabinoside, but cord formation is not affected by the addition of this agent.

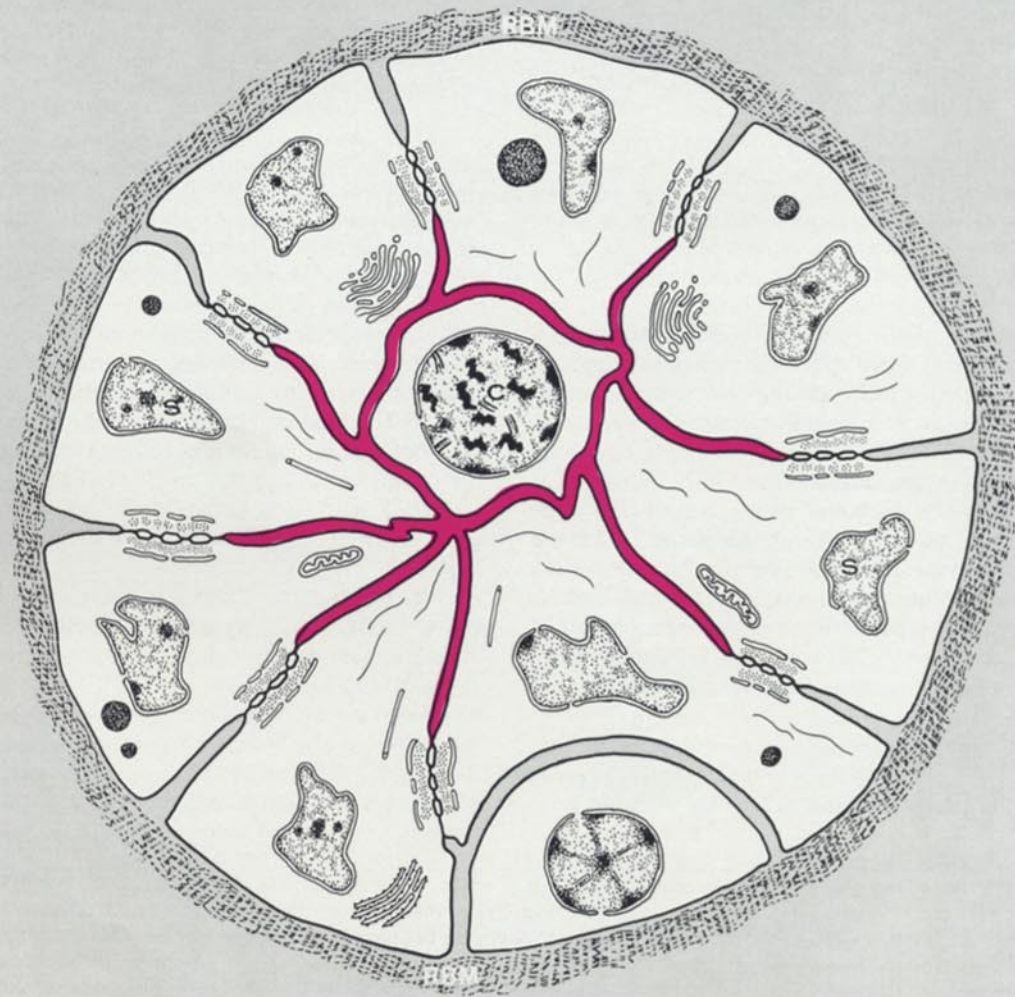
Protein Secretion

TOTAL PROTEIN: Sertoli cells grown on plastic secreted into the medium $\sim 40 \mu\text{g}$ protein/ 10^5 cells, every 3.5 d, during a 2-wk culture period (Fig. 6). On Sertoli-myoid matrix, this figure is $\sim 85 \mu\text{g}/10^5$ cells, and on RBM the figure averaged $\sim 120 \mu\text{g}/10^5$ cells. In general, the amount of total protein in the culture media from cells grown on either Sertoli-myoid matrix or RBM was two to three times, respectively, that in media obtained from cells grown on plastic. On RBM, Sertoli

FIGURE 3 (a) Phase-contrast micrograph of an in vitro Sertoli cell cord (CORD) which has formed from dissociated Sertoli cells cultured for 24 h within RBM. $\times 250$. (b) A light micrograph showing a cross-section through an in vitro Sertoli cell cord cultured for 14 d. Note the peripheral location of most Sertoli cell nuclei (S). Two germ cells (C) can be seen within the cord, one of which is a pachytene spermatocyte (star; see Fig. 4*a*). A degenerating figure can also be seen within the cord (arrowhead). RBM surrounds the cord. $\times 1,000$.



5a



5b

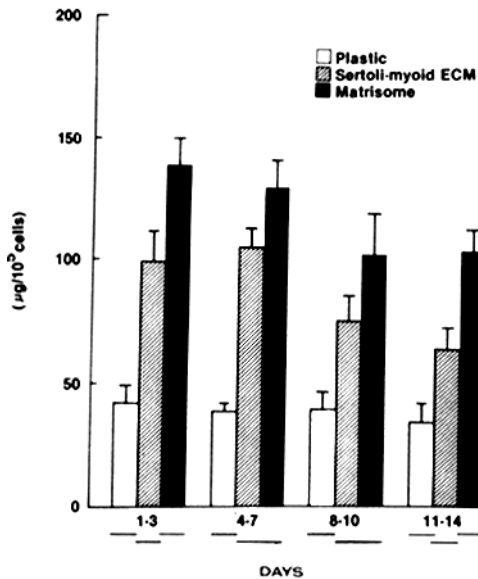


FIGURE 6 Total protein secretion. Total protein in aliquots of culture medium from Sertoli cells grown on different substrates was measured. Data are presented as the mean \pm SD for three separate experiments. Total protein in micrograms per 10^5 cells is represented on the ordinate, and sample times are indicated on the abscissa. Media were changed at 3, 7, 10, and 14 d during a 2-wk culture. At these times the spent media were collected for protein analysis. Any two means within a sample period (for example 1-3 d is a sample period) not underscored by the same line are significantly different at $P < 0.01$. Any two means underscored by the same line are not significantly different.

cell protein production was $\sim 30\%$ greater than that by cells grown on Sertoli-myoid matrix throughout the 2 wk of culture. Culture media incubated with either Sertoli-myoid matrix or RBM, without cells, showed no detectable increase in protein from values obtained for SFDM alone.

ABP: Sertoli cells grown on plastic secrete a stable level of ABP (145 ng/ 10^5 cells per 3.5 d) throughout the 2-wk culture period (Fig. 7). ABP secretion on the two matrix substrates averaged 270 ng/ 10^5 cells on Sertoli-myoid matrix and 390 ng/ 10^5 cells on RBM. Culture media incubated with either matrix substrate, without cells, showed no detectable ABP.

TRANSFERRIN: Transferrin secretion by Sertoli cells was also significantly greater on both Sertoli-myoid matrix (625 ng/ 10^5 cells per 3.5 d) and RBM (560 ng/ 10^5 cells) than on plastic (390 ng/ 10^5 cells) (Fig. 8). Transferrin levels stayed relatively stable on all substrates throughout the culture. There did not appear to be a significant difference in transferrin secretion between RBM and Sertoli-myoid matrix. Culture

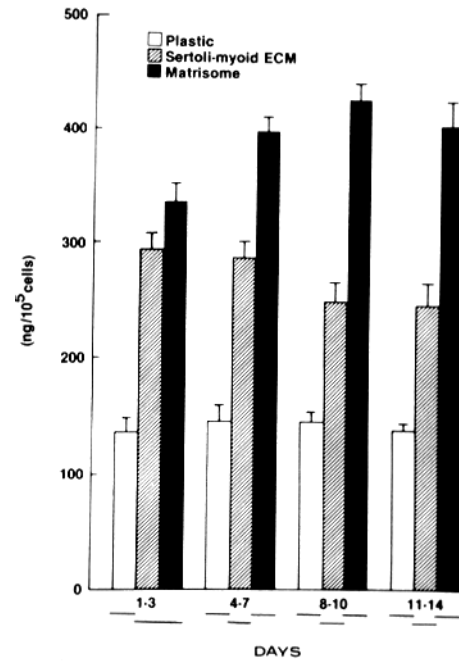


FIGURE 7 ABP secretion. ABP (nanograms per 10^5 cells) was measured, in aliquots (10 μ l) of medium from Sertoli cells grown on different substrates, using radioimmunoassay. Data are presented as the mean \pm SD for three separate experiments. Media were collected as described in Fig. 6. Any two means within a sample period not underscored by the same line are significantly different at $P < 0.01$. Any two means underscored by the same line are not significantly different.

media incubated with either matrix substrate, without cells, showed no detectable transferrin.

[³⁵S]METHIONINE-LABELED PROTEINS: The total proteins secreted by Sertoli cells, grown on plastic and RBM substrates in the presence of [³⁵S]methionine, were analyzed. SDS PAGE indicated that most of the proteins synthesized by the cells on these substrates appear similar (Fig. 9, lanes 1 and 2). In the molecular weight range of 140,000-300,000, however, significant differences are observed (Fig. 9, lanes 1 and 2). The material migrating at M_r 140,000-160,000 comigrates with authentic type I collagen standards, is selectively susceptible to collagenase digestion (Fig. 9, lane 3), and is pepsin resistant (data not shown). Therefore, the major collagen produced by these cells grown on RBM is type I.

[³H]THYMIDINE LABELING: The mitotic activity of Sertoli cells grown on the three substrates was measured (Table I). At day 4, Sertoli cells on plastic had a low mitotic index (0.68%) which was, nevertheless, significantly higher

FIGURE 5 (a) This schematic diagram of a polarized monolayer of Sertoli cells growing on top of a thin layer of RBM demonstrates how the culture medium (grey stipple) has equal access to both the apical and basal regions of the cells. Germ cells (C) are maintained in the basal region in these cultures, beneath the Sertoli-Sertoli tight junctions, and have direct access to the culture medium that flows through the RBM. No compartmentation exists in these cultures. (b) This schematic diagram of a cross-section through an *in vitro* cord grown within RBM demonstrates that a dual environment is established in these cultures. The Sertoli-Sertoli tight junctions create a central, or adluminal, compartment (color) distinct from the peripheral, or basal, compartment beneath the junctions. The culture medium (grey stipple) has free access to the peripheral compartment by filtering through the RBM. Early germ cells (spermatogonia; C) are found in this region and are directly exposed to the culture medium. The culture medium does not have free access to the central region. The milieu of this region (color) is controlled by the Sertoli cells and represents a composite of Sertoli cell secretion and compounds in the medium selectively transported by the Sertoli cells. Spermatocytes (C) that have developed from spermatogonia are found exclusively in this central (adluminal) compartment.

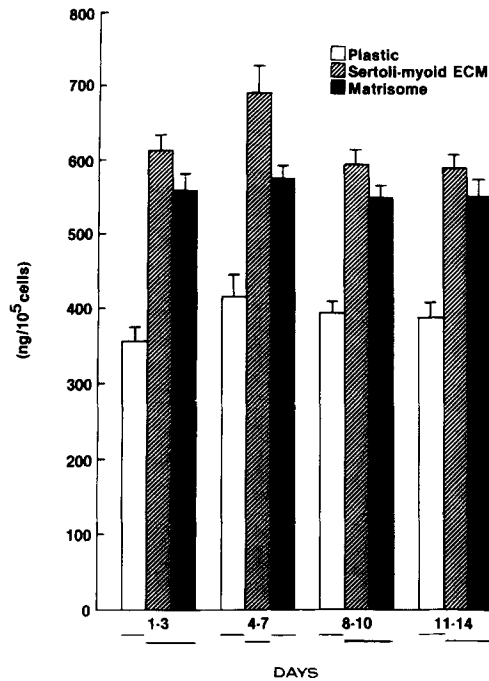


FIGURE 8 Transferrin secretion. Transferrin (nanograms per 10^5 cells) was measured by radioimmunoassay in aliquots ($10 \mu\text{l}$) of medium from Sertoli cells cultured on different substrates. Data are presented as the mean \pm SD for three separate experiments. Media were collected as described in Fig. 6. Any two means within a sample period not underscored by the same line are significantly different at $P < 0.01$. Any two means underscored by the same line are not significantly different.

than that of cells on Sertoli-myoid matrix (0.37%) or RBM (0.29%). By 6 d in culture, Sertoli cells on plastic and Sertoli-myoid matrix still showed [^3H]thymidine uptake, whereas those on RBM did not.

DISCUSSION

In this report, we demonstrate that the substrate upon which immature Sertoli cells are cultured dramatically influences their phenotype. Both morphological and biochemical data suggest that Sertoli cells grown as polarized monolayers on top of an RBM differentiate to provide a more functional representation of their in vivo counterparts than has been previously described. In addition, Sertoli cells embedded within an RBM gel, rather than grown on top of a thin layer of the RBM, undergo a dramatic morphogenesis to organize into cords that closely resemble the original testicular cords from which the cells were dissociated. Germ cells within the in vitro cords can differentiate, probably due to the compartmentalization of the cords by Sertoli-Sertoli tight junctions.

Other studies have demonstrated that co-culture of Sertoli and peritubular myoid cells can provide the Sertoli cells with information necessary for some degree of differentiation (12, 13, 17, 27). Sertoli cell morphology, survival in culture, and ABP secretion were all enhanced in these cultures. These experiments also suggested that some form of direct cooperation between Sertoli and myoid cells was necessary for the aggregation of Sertoli cells and the formation of a basal lamina (17, 27).

It was subsequently demonstrated that in the absence of myoid cells, extracellular matrix alone can provide Sertoli

cells with the appropriate stimulus for some degree of differentiation in vitro (18, 26, 28). A hierarchy seems to exist in the abilities of these various extracellular matrices to influence the differentiation of Sertoli cells. An extracellular matrix produced by a peritubular myoid cell line has been demonstrated to specifically increase ABP, but not transferrin, secretion by Sertoli cells in culture (18). The morphology of Sertoli cells on type IV collagen and laminin (26) was considerably

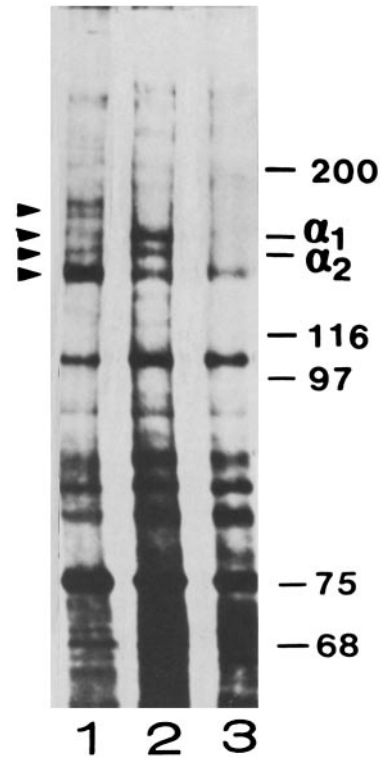


FIGURE 9 [^{35}S]Methionine labeled proteins secreted into culture media by Sertoli cells between days 5 and 6 of culture were analyzed by fluorography of SDS PAGE gels (6%). Samples (40,000 cpm/lane) were reduced with dithiothreitol before analysis. Lane 1, proteins from Sertoli cells grown on plastic; lane 2, proteins from Sertoli cells grown on RBM; lane 3, as lane 2, but the sample was incubated with purified bacterial collagenase as described in Materials and Methods. Numbers on the right correspond to the mobility of the following molecular weight standards: myosin (200,000), β -galactosidase (116,000), phosphorylase B (97,000), and bovine serum albumin (68,000). α_1 and α_2 indicate the mobility of the α_1 - and α_2 -chains of reduced acid-soluble bovine skin type I collagen. Arrowheads at the left indicate several bands that are differentially secreted by Sertoli cells on the two different substrates (lanes 1 and 2). Note that cells grown on RBM produce collagenase-sensitive proteins (lane 3) that correspond in molecular weight to the α_1 - and α_2 -chains of type I collagen (lanes 2 and 3).

TABLE I. Mitotic Indices of Sertoli Cells

Substrate	Days after plating	
	4	6
	%	
Plastic	0.68	0.13*
Sertoli-myoid matrix	0.37	0.10*
RBM	0.29	0.01*

Values are expressed as the mean \pm SD of percent labeled cells counted from 100 random fields on four separate coverslips for each substrate.

* $P < 0.01$ for all, except between day 6 on plastic and Sertoli-myoid matrix.

* No labeling recorded.

better than that of Sertoli cells on plastic. However, the Sertoli-myoid matrix used in the present study provides an even more suitable substrate, in that more Sertoli cell polarity and germ cell retention are achieved. A seminiferous tubule biomatrix extract (28) also provides an excellent substrate for Sertoli cell growth. Unfortunately, both Sertoli-myoid matrix and seminiferous tubule biomatrix are unable to maintain cell polarity in long-term culture, since the cells eventually spread out and resemble those grown on plastic.

RBM currently offers the best environment on which to grow Sertoli cells. Other reports, including our own, have shown that a variety of extracellular matrix components dried onto plastic cause improvement in some differentiated Sertoli cell functions in culture (18, 26, 28). However, Sertoli cell morphology in these cultures does not resemble that of the *in vivo* Sertoli cells. Since the highly specialized morphology of the Sertoli cell is undoubtedly essential to germ cell development, Sertoli cells growing on RBM represent a significant improvement in the culture system.

Because RBM is a gel, its porosity allows percolation of medium to the base of the cells (Fig. 5*a*). We believe that attachment factors in this matrix, as well as accessibility of nutrients to the base of the cells, are instrumental in preventing cell spreading and thus in maintaining a high local cell density. Sertoli cell aggregates plated on top of RBM rapidly reorganize to form very tall polarized cells each with basal contact to the substrate and an apical surface open to the medium. Their elaborate cytoskeleton and the lack of significant lateral spreading ensures that they maintain this extraordinary polarity. When Sertoli cells are grown within RBM gels the cells are also highly polarized; however their apical surface is not open to the medium but projects into the center of the cordlike structures (Fig. 5*b*).

The formation of Sertoli-Sertoli tight junctions is an important morphological expression of functional differentiation *in vitro* (23). The blood-testis barrier is not established *in vivo* until day 15-18, when the tight junctions are formed (30). Since the cultured cells in our study were isolated from 10-d-old rats, we would have anticipated tight junction formation within the first week of culture. This was indeed the case for Sertoli cells grown on top of RBM, as polarized monolayers, as well as within RBM gels, grown as cords.

Sertoli-Sertoli tight junctional complexes formed in both of these cell culture systems were consistently found between adjacent Sertoli cells and displayed the unique features described for *in vivo* Sertoli junctions (2). The junctional complexes were invariably basally located and up to 15 μm long. Solari and Fritz (23) have reported the formation of apically located tight junctions 0.5 μm long by Sertoli cells from 10-d-old rats cultured on plastic, while Tung and Fritz (28) have demonstrated the formation of basally located tight junctions in Sertoli cells from 20-d-old rats cultured on a seminiferous tubule biomatrix. However, in our study, Sertoli cells cultured on plastic, testicular biomatrix (unpublished observation), or Sertoli-myoid matrix did not demonstrate consistent tight junction formation, and when present, these tight junctions were generally apically located.

ABP has long been recognized as a marker of Sertoli cell function. In the present study, both ABP secretion and germ cell retention were increased in Sertoli cell cultures grown on matrix substrates. This is interesting in light of the recent work of Galdieri et al. (5) which showed that ABP secretion

by Sertoli cells grown on plastic was increased when purified populations of particular germ cell stages were added. Although their study did not include a purified population of spermatogonia (the only germ cell type present in our monolayer cultures), it is possible that the increase in ABP secretion by Sertoli cells that we note when they are grown on matrix substrates may be, in part, a direct reflection of their enhanced capacity to retain spermatogonia, as well as a direct response to their extracellular matrix substrate. Like Mather et al. (18), we find that transferrin secretion was only marginally increased when Sertoli cells were grown on matrix substrates instead of plastic, whereas ABP secretion was increased several times. Furthermore, secretion rates of both transferrin and ABP by Sertoli cells grown on RBM are approximately twice that reported for Sertoli cells in the literature (21, 22).

In response to RBM, Sertoli cells secrete putative type I collagen. This protein is selectively susceptible to collagenase, co-migrates with authentic type I collagen standards, and is pepsin resistant. In addition, Sertoli cells grown on permeable supports (which are impregnated with RBM) deposit an extracellular fiber with 67-nm repeating cross-striations identical to those seen in native type I collagen (unpublished data).

Griswold et al. (8) reported that Sertoli cells cultured at high densities on plastic synthesized less DNA than did low density cultures. Tung and Fritz (28) hypothesized that the diminished rate of DNA synthesis by Sertoli cells growing on a testicular extracellular matrix extract was related to the high cell density maintained by Sertoli cell aggregates cultured under these conditions. Tritiated thymidine labeling of cells grown on RBM, Sertoli-myoid matrix, and plastic seems to confirm these data. The degree to which Sertoli cells spread on each of these three substrates is directly related to their rate of mitosis. Cells do not spread readily on RBM and thus their mitotic index is lowest.

A combination of substrate composition, cell density, and basal diffusion of medium appears necessary for maintenance of highly differentiated monolayers of Sertoli cells *in vitro*. RBM permits a dramatic improvement of cell polarity and metabolism. However, growing Sertoli cells on top of RBM fails to preserve one additional characteristic of the *in vivo* Sertoli cell. Sertoli cells *in vivo* establish a dual environment within the seminiferous tubule, each with a distinct milieu. The Sertoli-Sertoli tight junctions create a blood-testis barrier which separates the basal and adluminal compartments of the seminiferous tubule (2). The basal compartment is in direct contact with the general circulation, whereas the adluminal compartment is subject largely to substances secreted or transported by the Sertoli cells. The compartmentalization of the seminiferous tubule is thought to be instrumental in the support role played by Sertoli cells in the spermatogenic process. The apical, or adluminal, environment *in vivo* supports the later stages of germ cell differentiation. This environment is missing in monolayer cultures. Even though characteristic tight junctions are formed by Sertoli cells grown on top of RBM, they fail to functionally compartmentalize the monolayer since culture medium has free access to both the basal and apical surfaces of the cells (Fig. 5*a*). Germ cells are maintained by Sertoli cells cultured as monolayers on RBM, but there is no progression of spermatogonia to later germ cells.

By increasing the thickness of the RBM gel and culturing Sertoli cells within rather than on top, we can modify the

organization of the cultured Sertoli cells (Fig. 5b). Sertoli cells grown within these thicker gels no longer form monolayers but organize to form an elaborate network of long anastomosing cords within the matrix, which closely resemble the original testicular cords from which they were dissociated. The Sertoli cells of the in vitro cords also demonstrate a highly differentiated and polarized morphology similar to that which they acquire in monolayer culture on RBM. Basal tight junctional complexes functionally compartmentalize the cords into basal and adluminal compartments. Thus a dual environment that appears essential for germ cell differentiation is established (Fig. 5b). By using testes from 10-d-old rats in our experiments, we can ensure that the germ cells contaminating the enriched Sertoli cell cultures have developed no further than type B spermatogonia. We have demonstrated for the first time that germ cell differentiation can progress (from spermatogonia to late pachytene spermatocytes) in primary cultures of dissociated Sertoli cells. This provides the first evidence that the blood-testis barrier is necessary for germ cell development. The degree of differentiation maintained by Sertoli cells grown on top of or within RBM indicates that these cultures will prove to be a valuable tool with which to dissect the complex interactions among Sertoli, myoid, and germ cells within the testis. In addition, we have now succeeded in growing polarized monolayers of Sertoli cells in cell culture chambers that allow experimental access to both luminal and basal compartments (Byers, S. W., M. A. Hadley, and M. Dym, manuscript submitted for publication).

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