# Cooperativity between Sertoli Cells and Peritubular Myoid Cells in the Formation of the Basal Lamina in the Seminiferous Tubule

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## INTRODUCTION

When Sertoli cell–enriched aggregates are plated on top of a layer of peritubular myoid cells, the properties of each of the two cell types are very different from those observed in cells in monoculture. In the co-cultured system, both cell populations survive for months in a serum-free medium. In contrast, peritubular myoid cells in monoculture detach from the plate within days when they are plated in the absence of serum, and primary cultures of Sertoli cells do not remain functional beyond three weeks. In the co-cultured system, Sertoli cells and peritubular cells interact, undergoing a morphological rearrangement to form a structure resembling the seminiferous tubule, and a basement membrane is laid down between the two cell types.[1] In addition, the production of androgen binding protein (ABP) by Sertoli cells is enhanced in the presence of peritubular cells, and ABP formation is maintained for longer periods in the co-cultured system.[1,2]

It appeared possible that separate extracellular matrix (ECM) components synthesized by Sertoli cells and peritubular myoid cells in co-culture may interact to permit the formation of a basement membrane that cannot be elaborated by either cell type alone. We offer the speculation that cooperativity between Sertoli and peritubular cells may be required for the synthesis of all ECM components needed for basal lamina organization and formation. In initial attempts to examine these possibilities, we have investigated the synthesis of specific ECM components by each cell type. In addition, we have explored the effects of various ECM components on the histotype of Sertoli cells and peritubular cells maintained in monoculture. Results to be presented demonstrate that the two cell types secrete different ECM components, and that the nature of the substratum upon which the cells are cultured greatly influences the histotype expressed.

## MATERIALS AND METHODS

Procedures for the preparation and culture of conventional rat Sertoli cell–enriched aggregates,[3,4] of hyaluronidase-treated purified Sertoli cell–enriched aggregates,[5] and of primary and secondary cultures of peritubular cells[1,4] were the same as those previously described. All cells were maintained and cultured in Eagle's

modified essential minimal medium (MEM), containing either no serum or 10% calf serum, (GIBCO, Grand Island, NY) as specified in the legends to figures. All cells were prepared from testes of 20-day-old Wistar rats unless specified otherwise.

We employed fluorescent microscopic procedures for the localization of laminin or fibronectin by indirect immunofluorescent techniques, using the same procedures as those described elsewhere.[5,6] Antisera employed included rabbit antiserum against laminin, kindly provided by Drs. George Martin and Hynda Kleinman (National Institutes of Health, Bethesda, MD); goat antiserum directed against human or rat fibronectin, purchased from Calbiochem (La Jolla, CA); and a mouse monoclonal IgG directed against porcine fibronectin, kindly provided by Dr. J. Aubin (University of Toronto, Toronto, Canada). Bulk adsorption techniques with lyophilized laminin and fibronectin were performed as described elsewhere,[5] and morphological techniques were the same as those previously employed.[5,7,8]

Techniques for the culture of cells on various substrata, including seminiferous tubule biomatrix (ST-matrix), were the same as those recently described.[9]

## RESULTS

### *Detection of Fibronectin and Laminin in Peritubular Boundary Tissue*

We employed indirect immunofluorescent microscopy to localize fibronectin and laminin in cryostat sections of testes from 20-day-old rats. Experiments with antisera to these glycoproteins revealed that fibronectin and laminin were each distributed primarily in or along the basal lamina of the seminiferous tubule boundary tissue (FIGURE 1). Neither of these ECM components was detectable within the luminal compartment. Antibodies against fibronectin and laminin preadsorbed with fibronectin or laminin, respectively, did not react. In contrast, fibronectin antibody preadsorbed with laminin, or laminin antibody preadsorbed with fibronectin, retained full activity (data not shown).

### *Fibronectin is Detectable in Cultures of Peritubular Cells but not in Cultures of Sertoli Cells*

Peritubular myoid cells in culture for 24 hr or longer have a positive reaction to fibronectin antibodies, whereas purified populations of Sertoli cells do not.[5] The nature of the fibronectin localization depends upon the culture conditions, with a fibrillar-like extracellular arrangement in preparations cultured in the presence of serum, and predominantly a perinuclear distribution in peritubular cells cultured in serum-free medium for four days (see FIGURES 2 and 3 of Tung *et al.*[5]). Primary cultures of peritubular cells show occasional areas free of fibronectin antibody-reactive material (FIGURE 2 A,B), associated with the inclusion of a few Sertoli cell aggregates in the preparation. In contrast, almost all peritubular cells in secondary cultures are positive for fibronectin (FIGURE 2 C and D).

To extend these observations obtained with indirect immunofluorescent microscopy, we have investigated the synthesis of fibronectin by cells cultured in medium containing [$^{35}$S]methionine. Proteins immunoprecipitated with fibronec-

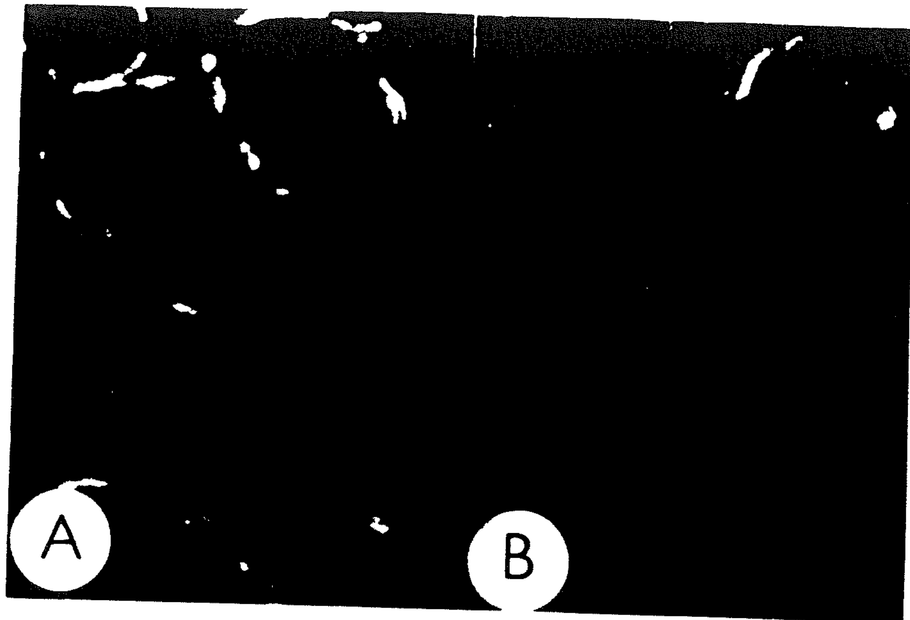


**FIGURE 1.** Immunofluorescent micrographs of cryostat sections of testis from a 20-day-old rat. (A) A section initially reacted with goat antiserum against human fibronectin (1 : 20), and then with FITC-conjugated rabbit anti-goat IgG (1 : 30 with PBS). (B) A section initially reacted with rabbit antiserum against laminin (1 : 10), followed by incubation with FITC-conjugated goat-anti-rabbit IgG (1 : 30). Immunofluorescence was not detected with antisera previously adsorbed with fibronectin or laminin, respectively (data not shown). (300 ×).

tin antibodies were subjected to slab gel electrophoresis. The gels were then fluorographed or they were stained with Coomassie blue. Peritubular cells secreted a labeled protein, immunoprecipitated with a fibronectin antibody, that had a molecular mass (220,000) indistinguishable from that of authentic fibronectin (FIGURE 3). In contrast, a protein having these characteristics was not detectable in secretions from Sertoli cell–enriched aggregates that had been treated with hyaluronidase (FIGURE 3).

Conventional Sertoli cell–enriched aggregates not treated with hyaluronidase during the isolation procedure are contaminated by peritubular cells (7.6 ± 0.9%). These cells readily proliferate when cultured in the presence of 10% calf serum unless an inhibitor of DNA synthesis is added.[10] After four days in medium containing serum but no inhibitor, the percentage of peritubular cells in conventional Sertoli cell–enriched aggregates increases to 23 ± 1.7%.[5] This is correlated with the presence of fibronectin in conventional Sertoli cell preparations, which are cultured in medium containing 10% calf serum (FIGURE 4 A and C). We have used the detectability of fibronectin as a criterion for improving procedures for isolating Sertoli cell–enriched aggregates free of containing peritubular cells, and have observed that treatment with hyaluronidase diminishes the number of peritubular cells in the preparation. Such purified Sertoli cell preparations have no

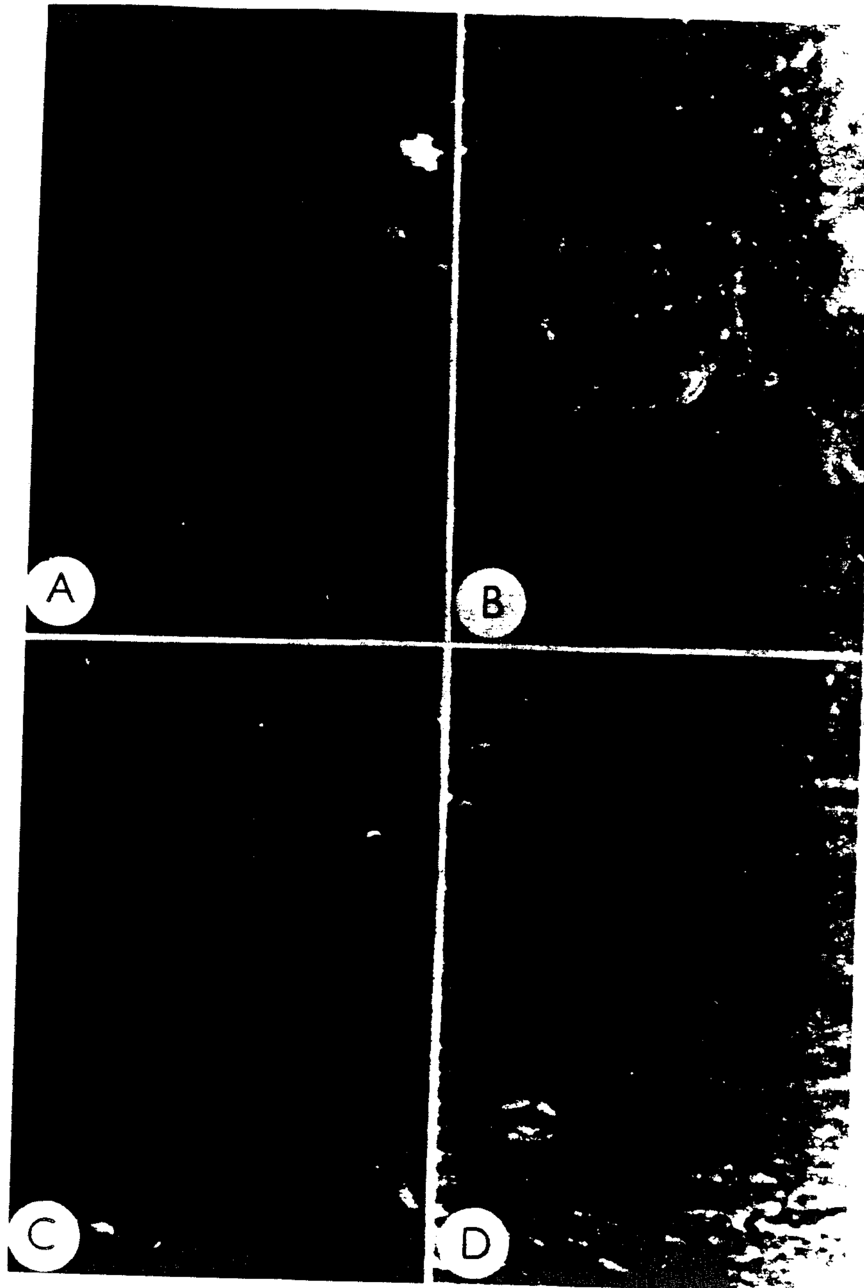
A
B
C
D

**FIGURE 3.** Electrophoretic analysis of radiolabeled proteins synthesized by cultures of Sertoli or peritubular cells, and immunoprecipitation by fibronectin antibody. An SDS 5 to 15% polyacrylamide gradient slab gel was either stained with Coomassie blue (lanes H to J), or radiolabeled proteins were fluorographed (lanes A through G). The fluorograph lanes are (A) peritubular cell–secreted radiolabeled proteins, (B) goat anti-rat fibronectin immunoprecipitate of peritubular cell–secreted proteins; (C) mouse anti-pig fibronectin immunoprecipitate; (D) control non-immune serum immunoprecipitates of peritubular cell–secreted proteins; (E) Sertoli cell–secreted proteins; (F) goat anti-rat fibronectin immunoprecipitate of Sertoli cell–secreted proteins; and (G) control non-immune immunoprecipitate of Sertoli cell–secreted proteins. (From Tung *et al.*[5] With protein lanes are: (H) rat fibronectin, (I) peritubular cell–secreted proteins; and (J) Sertoli cell–secreted proteins. (From Tung *et al.*[5] With permission from *Biology of Reproduction*.)

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**FIGURE 2.** Immunofluorescent micrographs (A and C) and phase-contrast micrographs (B and D) of representative fields of primary peritubular cell cultures (A and B) and secondary cultures (C and D). The primary culture (A,B) had been maintained on glass coverslips in modified MEM containing 10% calf serum for 6 days. Cells were then fixed (3% paraformaldehyde in $Ca^{2+}$- and $Mg^{2+}$-free Hanks' buffer for 20 min at room temperature, followed by immersion in acetone at −10°C for 7 min) and reacted with goat antiserum against fibronectin (1 : 25). Arrows in (A) point to a fibronectin-negative area associated with unidentified cell contaminants, possibly Sertoli cells. The secondary culture (C,D) had been prepared by plating trypsinized primary cultures of peritubular cells onto glass coverslips, and then treating these cells (after maintenance for 6 days in culture) in a manner identical to that described above for cells in primary culture. (750 ×).

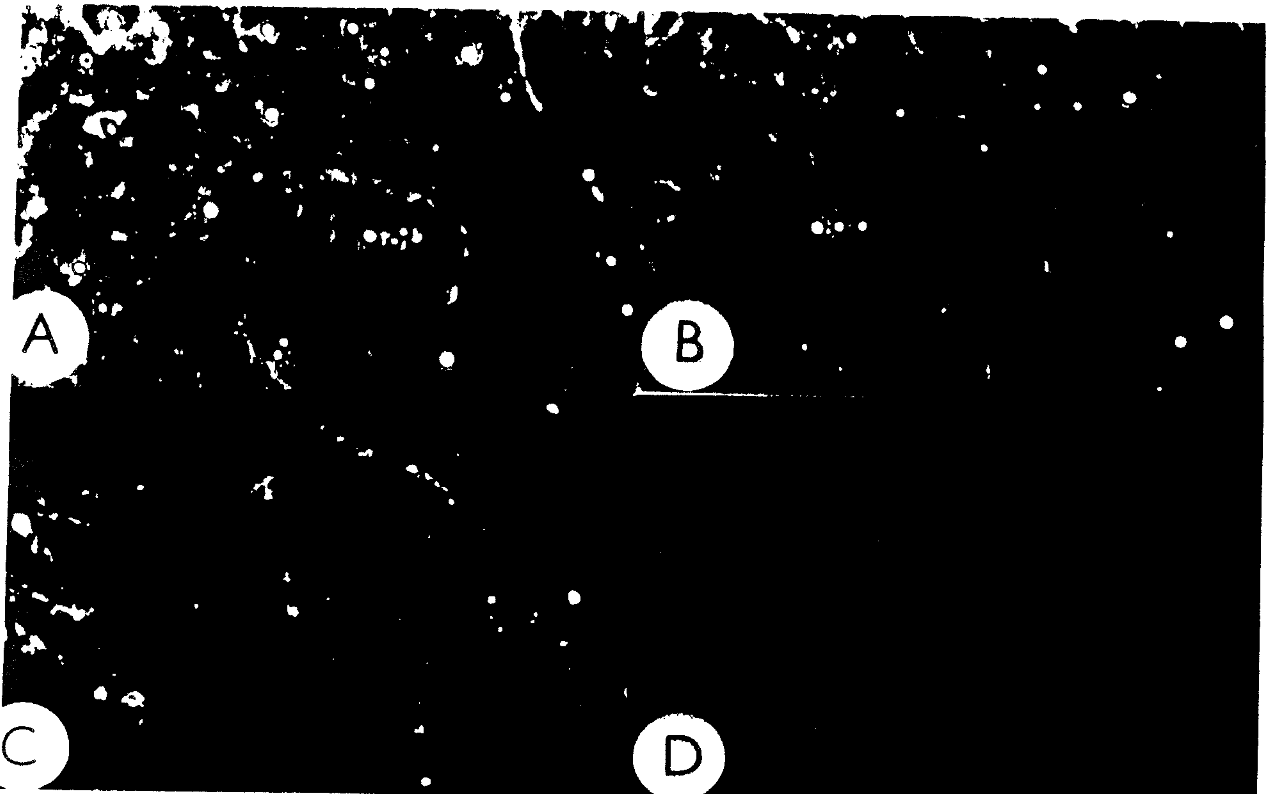


**FIGURE 4.** Phase-contrast micrographs (A,B) and immunofluorescent micrographs (C,D) of confluent cultures of Sertoli cells. (A) and (C) show a representative field of a dense culture (1,500 aggregates/100 mm$^2$) from a conventional Sertoli cell–enriched preparation not subjected to hyaluronidase treatment. (B) and (D) show a representative field of a comparably dense culture from a purified Sertoli cell–enriched preparation that had previously been digested with hyaluronidase, as described in METHODS. All cells had been cultured for 6 days in modified MEM containing 10% calf serum, fixed, and reacted with fibronectin antiserum as described in the legends to FIGURES 1 and 2. Similar observations have been made in cells plated at lower density (200 aggregates/100 mm$^2$). (750 ×). (From Tung *et al.*[5] With permission from *Biology of Reproduction*.)

detectable fibronectin, even when cultured for four days in medium containing 10% calf serum (FIGURE 4 B and D), and the number of microscopically recognizable peritubular cells is less than 1%.[5]

These data demonstrate that fibronectin is a suitable marker for the presence of peritubular cells in testicular cell cultures. Determination of rates of fibronectin synthesis may be expected to provide a more quantitative measure of the degree of contamination of Sertoli cell–enriched preparations by peritubular cells.

### *Laminin is Detectable in Cultures of Sertoli Cells but not in Cultures of Peritubular Cells*

Newly isolated aggregates of conventional or purified Sertoli cell–enriched preparations did not initially react with laminin antiserum (data not shown). Shortly after conventional Sertoli cell–enriched aggregates attached to the polystyrene surface (3 to 4 hr after plating), cells within the aggregate began to migrate, frequently assuming a fibroblast-like appearance as they spread beyond the initial aggregates. These rapidly migrating cells failed to show any reaction to laminin antibodies. In contrast, cells within the aggregate reacted positively with laminin antibodies. The strongest reactions appeared in cells around the periphery of the aggregates (FIGURE 5 A and C). The location of fluorescence in the indirect immunofluorescent microscopic assay for laminin precisely followed the shape of the colonies of aggregated Sertoli cells, and this remained evident in purified Sertoli cell–enriched preparations maintained in culture for periods up to 14 days (data not shown). Peritubular cell preparations in culture did not react with laminin antibodies (FIGURE B and D). Laminin was undetectable in peritubular or interstitial cells maintained in culture for periods up to 10 days.

### *Seminiferous Tubule Biomatrix Promotes Rat Sertoli Cell Histotypic Expression* in Vitro

We have investigated the possible effects of altering the substratum upon which Sertoli cell–enriched preparations are plated on the properties of these cells in culture. Various substrata examined included uncoated polystyrene, polystyrene coated with seminiferous tubule biomatrix (ST-biomatrix), and polystyrene coated with Type I collagen or polylysine. Sertoli cells maintained their normal *in vivo* histotype best when cultured on the ST-biomatrix (FIGURES 6 and 7). The most striking differences consisted of the taller columnar-shaped appearance of Sertoli cells maintained on ST-biomatrix. These cells had an average height of 8.7 $\mu$M, whereas Sertoli cells cultured on uncoated plastic for 6 days under otherwise comparable conditions had an average thickness of only 2 $\mu$m (FIGURE 6). In addition, basolateral tight junctions were maintained in cells in culture on the ST-biomatrix but not on the other substrata employed (FIGURES 6 and 7). The percentage of cells surviving at 21 days after plating was increased from about 25% of cells plated on polylysine-coated polystyrene to 80% of cells plated on ST-biomatrix.[9]

Peritubular cells cultured on each of these substrata in MEM containing 10% calf serum readily spread to form monolayers. We are currently investigating the differences in morphology of peritubular cells maintained on ST-biomatrix, Type I collagen, or uncoated plastic (Tung and Fritz[9] and unpublished observations).

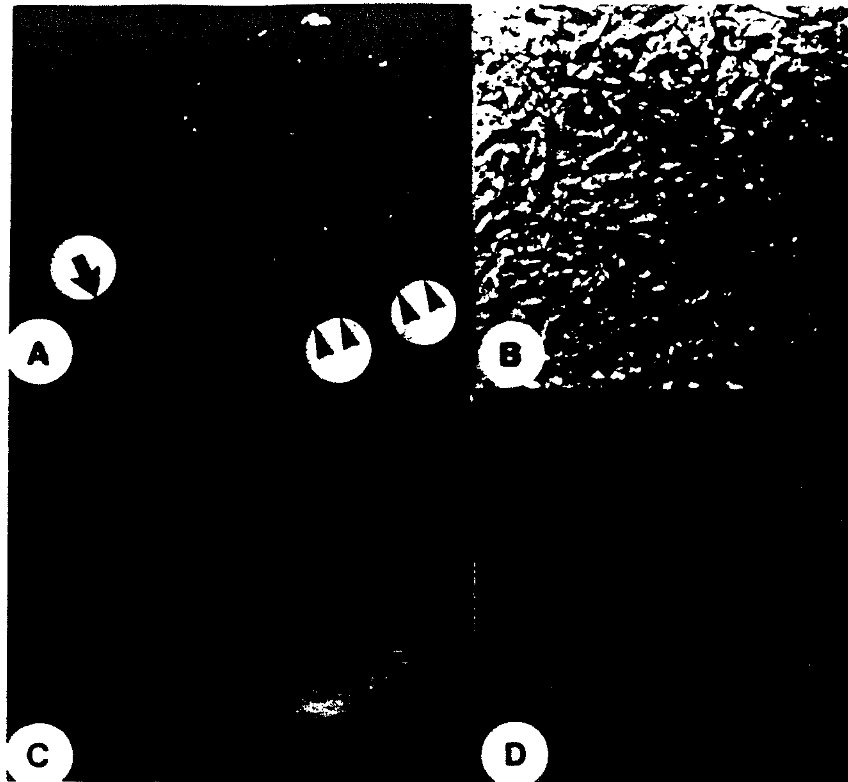


**FIGURE 5.** Phase-contrast micrographs (A,B) and immunofluorescent micrographs (C,D) of Sertoli cell–enriched preparations (A,C) and peritubular cells (B,D) reacted with rabbit antiserum against laminin (1 : 15). (A) and (C) show a representative aggregate of a conventional Sertoli cell–enriched preparation maintained for 6 hr in modified MEM containing 10% calf serum. The preparation was fixed 6 hr after plating and incubated with laminin antiserum. Note that only the fibroblast-like cells in (A) (arrows) that have migrated away from the Sertoli cell aggregate are negative for laminin-dependent immunofluorescence (C). (B) and (D) show a representative portion of a primary culture of peritubular cells maintained for 6 days in modified MEM containing 10% calf serum, and demonstrate the absence of laminin-dependent immunofluorescence in peritubular cells (D). Sertoli cells at the periphery of the aggregate (C) have strongest reactions with laminin antibody. (350 × figure reduction, 70%).

## DISCUSSION

Data presented indicate that fibronectin and laminin are distributed primarily in or along the basal lamina of the seminiferous tubule (FIGURE 1), and that these ECM components are most probably derived from different cell types. Sertoli cells in culture synthesize laminin but not fibronectin, while peritubular cells in culture produce fibronectin but not laminin (FIGURES 2–5). In experiments in progress, we have observed other differences between the two cell types with respect to the

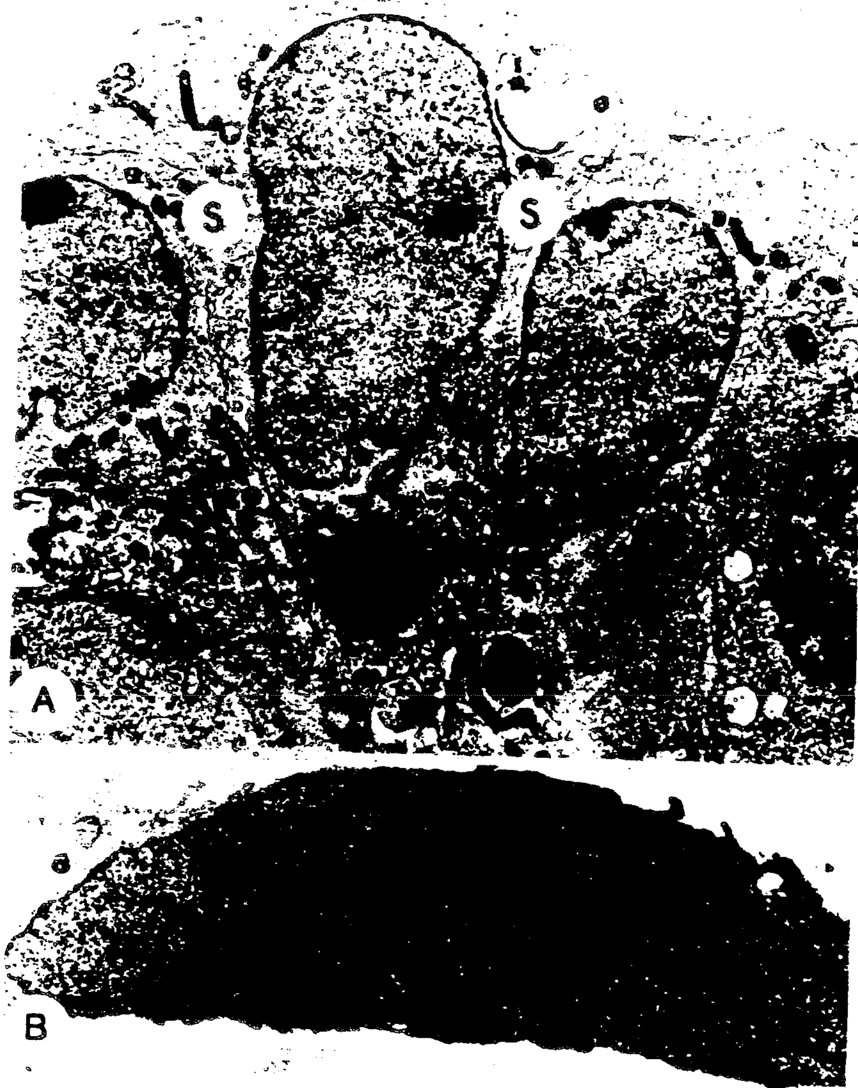


**FIGURE 6.** Transmission electron micrographs of vertical sections of Sertoli cells prepared from testes of 20-day-old rats, and maintained for 6 days in serum-free MEM. In (A), the substratum for the cells was ST-biomatrix, and in (B) the substratum was uncoated polystyrene. Note taller cell shape of cells cultured on ST-biomatrix (average thickness of 8.7 μm) than of cells cultured on plastic (average thickness of 2.0 μm) S represents adjacent Sertoli cells, and the arrow indicates junctional complex in basolateral region (× 18,400, figure reduction, 95%). (From Tung & Fritz.[9] With permission from *Biology of Reproduction*.)

**FIGURE 7.** Transmission electron micrograph of a vertical section of Sertoli cells prepared from testes of 20-day-old rats, and maintained for 6 days in serum-free MEM. Note presence of abundant microtubules and microfibrils, oriented vertically (× 19,600, figure reduction, 95%).

nature of ECM components synthesized. For example, each of the two cell types synthesize proteoglycans, but the nature of the proteoglycans is different (Skinner and Fritz, in preparation). The proteoglycans formed by Sertoli cells in culture are of different molecular mass than those synthesized by peritubular cells. We think it highly probable that the formation of the basement membrane that takes place in co-cultures of Sertoli cells and peritubular cells[1] is dependent upon contributions of different ECM components by each cell type. The physiological significance of these interactions in basal lamina formation and maintenance *in vivo* remains to be determined. The data, however, are consonant with the speculation that the basal lamina of the seminiferous tubule boundary tissue is deposited as a consequence of cooperativity between Sertoli cells and peritubular cells.

Hyaluronidase treatment of Sertoli cell–enriched aggregates greatly diminishes the number of contaminating peritubular cells in the preparation. Addition of this step to the isolation procedure permits the culture of purified populations of Sertoli cell aggregates in medium containing 10% calf serum without the overgrowth of peritubular cells that otherwise would occur.[5,9] Some of the proteins reported to be synthesized by conventional preparations of Sertoli cell–enriched preparations cultured in the presence of serum could have been produced by peritubular cells. The synthesis of fibronectin provides a suitable marker to evaluate this possibility.

The functions of the basal lamina in the seminiferous tubule are not established. It provides a structural support that separates the basal surfaces of Sertoli cells from adjacent peritubular cells, but it may also have other functions. The normal *in vivo* histotype of Sertoli cells is remarkably well maintained in cells plated on seminiferous tubule biomatrix as substratum. Sertoli cell aggregates plated on this substratum retain a cuboidal to columnar shape, with numerous and complex surface projections. They manifest a normal polarity as indicated by the presence of tight junctional complexes in the basolateral regions; and they have an abundance of microtubules and microfibrils in vertical bundles perpendicular to the basal surface of the columnar cells (Tung and Fritz,[9] and FIGURES 6 and 7). The ST-biomatrix was observed to be superior to other substrata examined (uncoated polystyrene or polystyrene coated with Type I collagen or polylysine) in maintaining the unique histotypic features of Sertoli cells.[9] These data suggest that the seminiferous tubule basal lamina may play an important role in orienting the structure of Sertoli cells, and thereby indirectly influencing the architecture of the seminiferous tubule. Further studies are in progress to determine factors that modulate the synthesis of specific ECM components by each cell type and to evaluate the control of the deposition of ECM components during the *in vitro* formation of the basal lamina–like structure. We are evaluating the hypothesis that Sertoli cells and peritubular cells act synergistically to generate the basal lamina of the seminiferous tubule.

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