Sertoli Cell Binding to Isolated Testicular Basement Membrane

George C. Enders,*‡ John H. Henson,§ and Clarke F. Millette*‡

*Laboratory of Human Reproduction and Reproductive Biology, ‡Department of Anatomy and Cellular Biology, and §Cell and Developmental Biology Program, Harvard Medical School, Boston, Massachusetts 02115

Abstract. We have examined the adhesion of primary Sertoli cells to a seminiferous tubule basement membrane (STBM) preparation in vitro. The STBM isolation procedure (Watanabe, T. K., L. J. Hansen, N. K. Reddy, Y. S. Kanwar, and J. K. Reddy, 1984, *Cancer Res.*, 44:5361–5368) yields segments of STBM that retain their histotypic form in both three-dimensional tubular geometry and ultrastructural appearance. The STBM sleeves contain two laminae: a thick, inner basal lamina that was formed in vivo between Sertoli cells and peritubular myoid cells; and a thinner, outer basal lamina that was formed between myoid cells and sinusoidal endothelial cells. Characterization by immunofluorescence and SDS PAGE revealed that the isolated STBM retained fibronectin, laminin, and putative type IV collagen among its many components. When the STBM sleeves were gently shaken with an enriched fraction of primary Sertoli cells, the Sertoli cells bound preferentially to the lumenal basal lamina at the ends of the STBM sleeves. Few Sertoli cells bound to either the outer basal lamina of the STBM sleeves or to vascular extracellular matrix material which contaminated the STBM preparation. 3T3 cells, in contrast, bound to all surfaces of the STBM sleeves. Pretreatment of the STBM sleeves with proteases, 0.1 M Na metaperiodate, 4 M guanidine HCl, or heating to 80°–90°C inhibited lumenal Sertoli cell binding, but binding was not inhibited by chondroitinase ABC, heparinase, hyaluronidase, or 4 M NaCl. The lumenal Sertoli cell binding occurred in the presence or absence of added soluble laminin or fibronectin. The addition of soluble laminin, but not fibronectin, restored random binding of Sertoli cells to trypsinized STBM sleeves. Our in vitro model system indicates that Sertoli cells recognize differences in two basal laminae produced in vivo on either side of myoid cells.

MOST multicellular organisms have substrates upon which their cells migrate, grow, and differentiate. This extracellular matrix is generally composed of glycoproteins, collagens, and proteoglycans, and influences a wide variety of cell functions (Kleinman et al., 1981; Reid et al., 1981; Bissel et al., 1982; Hay, 1981). Cell attachment and adhesion, for example, are often dependent upon, or modulated by, constituents of the extracellular matrix.

A variety of in vitro model systems have been used to study cell attachment and adhesion. Some investigators have examined the adhesion of primary or transformed cell lines to specific extracellular matrix components plated on plastic culture dishes (Johansson et al., 1981; Pierschbacher, et al., 1981; Yamada, 1983). Others have analyzed the adhesion of cells to extracellular matrices made in vitro, e.g., extracellular matrices produced by corneal endothelial cells (Greenburg and Gospodarowicz, 1982; Fridman et al., 1985) or human amnion epithelial cells (Aplin et al., 1984). Since in vivo extracellular matrices are thought to be produced by the interaction of at least two different cell types (Farquhar, 1981; Sariola et al., 1984; Abrahamson, 1985; Skinner et al., 1985), the use of primary extracellular matrix isolated from in vivo sources should offer advantages as a model system. Primary extracellular matrix preparations isolated from various organs have been used to study cell attachment and adhesion, but often these preparations have involved homogenization of the extracellular matrix, destroying its architecture (Rojkind et al., 1980; Tung and Fritz, 1984).

In this paper we examine the adhesion of Sertoli cells to an extracellular matrix preparation isolated from rat testis according to the procedures of Reddy and co-workers (Reddy et al., 1983; Watanabe et al., 1984). This method yields short sleeves of seminiferous tubule basement membrane (STBM)[1] which retain the in vivo histotypic form, both in their three-dimensional sleeve-like geometry and ultrastructural appearance. When an enriched fraction of primary Sertoli cells is isolated and gently shaken with a suspension of STBM sleeves, the cells preferentially adhere to the lumenal surface exposed at the ends of the STBM tubes. This lumenal surface represents the normal in vivo attachment site for Sertoli cells. Other cell types examined, including 3T3 cells, bound to all exposed surfaces of the STBM sleeves.

While this in vitro model system does not readily lend itself to quantitative analysis due to the unusual asymmetric nature of the adhesion, it offers a number of specific advan-

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1. *Abbreviations used in this paper:* DME/F-12, Dulbecco's modified Eagle's medium/Nutrient Mixture F-12; FCS, bovine calf serum; STBM, seminiferous tubule basement membrane.

tages for the investigation of cell–matrix interactions over other in vitro assay systems. Most importantly, the physiological geometry of the isolated extracellular matrix material allows one to determine that the specificity of binding in vitro reflects that seen in vivo. Preliminary reports of the work have been presented in abstract form (Henson et al., 1984; Enders et al., 1985).

Materials and Methods

Preparation of STBM Sleeves

Preparation of the STBM sleeves was based on the work of Reddy and co-workers (Reddy et al., 1983; Watanabe et al., 1984). Adult albino rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were anesthetized with ether and killed by cervical dislocation. The testes were removed and decapsulated, the major testicular artery was excised, and the testes were placed into enriched Kreb's Ringer bicarbonate buffer (as formulated by Bellvé et al., 1977) on ice. The seminiferous tubules were then sliced into ∿2-mm segments with razor blades. The cut segments were washed in enriched Kreb's Ringer bicarbonate buffer and pelleted by centrifugation in polypropylene centrifuge tubes at 200 g for 5 min. The pelleted tubules were then pressed between two parafilm-covered glass plates to squeeze out cellular material. The pressed tubules were washed again in enriched Kreb's Ringer bicarbonate buffer and resuspended into 20 ml deionized water, 0.05% wt/vol sodium azide per testis, and gently shaken on ice for 1–1.5 h in polypropylene tubes. Subsequently, tubules were pelleted at 1,000 g for 5 min and resuspended using a polypropylene pipette in 1 M NaCl, 200 K U/ml DNase (Sigma Chemical Co., St. Louis, MO), 2 mM phenylmethylsulfonyl methoxide, and gently shaken for 1.5–2.0 h at room temperature. The tubules were then pelleted and resuspended in 1–1.5% wt/vol sodium deoxycholate in water (pH 7.8 ± 0.2) and shaken on ice for an additional 1–1.5 h. The resulting STBM sleeves were washed twice in sterile Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 with L-glutamine, 15 mM Hepes buffer, 10 mg/liter gentomycin (DME/F-12; Gibco, Grand Island, NY). For some experiments, a mixture of 100 U penicillin, 0.25 μg Fungizone, 100 mcg streptomycin/ml (Gibco) was also added. Sleeves were then resuspended in DME/F-12 plus one of the following: (a) 10% bovine calf serum (FCS, K.C. Biologicals, Inc., Lenexa, KA), (b) 1–5 mg/ml bovine serum albumin (BSA, essentially globulin-free, Sigma Chemical Co.), and (c) 1 mg/ml lytic enzymes (see below). STBM sleeves were used the same day as their preparation or were stored at 4°C until used.

Immunocytochemical Localization

Throughout the immunocytochemical localization procedures the STBM sleeves were maintained in DME/F-12 containing 1–5 mg/ml BSA. Rabbit antisera to fibronectin (E. Y. Laboratories, Inc., San Mateo, CA) and to laminin (E. Y. Laboratories and a gift from Dr. Hynda Kleinman) were diluted 1:100–1:500 in the same medium and incubated with STBM sleeves overnight at 4°C with gentle agitation. The STBM sleeves were washed three times in medium and exposed to fluorescein-labeled goat anti–rabbit IgG (Calbiochem-Behring Corp., La Jolla, CA), diluted 1:100–200 in medium, for 3–4 h at 4°C. Tubules were then washed three times in medium and examined by epifluorescence using a Zeiss Photomicroscope III. Control experiments included the use of nonimmune rabbit serum, the addition of 30% FBS to antifibronectin, and 100 μg of laminin to antilaminin antisera (Collaborative Research, Inc., Waltham, MA), before subsequent incubation with the STBM sleeves. Photographs were taken using automatic exposure and Kodak Tri-X film at ASA 1600. Controls were manually exposed for equivalent times. Antisera were also examined by immunodiffusion against STBM, laminin, fibronectin, type IV collagen (Bethesda Research Laboratories, Gaithersburg, MD or Collaborative Research, Inc.) and normal rat serum.

SDS PAGE

STBM samples in deionized water were mixed with equal volumes of 2X sample buffer (Laemmli, 1970) containing 80 mM dithiothreitol instead of β-mercaptoethanol. Samples were boiled for 5 min and run on 4–10% acrylamide gradients with 3% acrylamide stacking gels. Laminin, type IV collagen, and molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were subjected to electrophoresis in adjacent lanes.

Isolation of Primary Sertoli Cells

Primary Sertoli cells were isolated from either 17–19-d-old CD-1 mice or 25-d-old albino rats (Charles River Breeding Laboratories, Inc.) according to one of the procedures of Mather and Phillips (1984). Briefly, decapsulated testes were placed into 1 M glycine, 2 mM EDTA, 20–40 KU/ml DNase (Sigma Chemical Co.), 0.2 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.) in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS), pH 7.2 ± 0.1. The testes were gently disrupted into tubules and free interstitial cells by pipetting through increasingly smaller diameter pipettes over a period of 15 min at room temperature. Tubules were freed of interstitial cells by allowing the tubules to settle at unit gravity and decanting the supernatant. Tubules were washed by resuspension in fresh DME/F-12 three times and then placed in 0.5 mg/ml collagenase/dispase (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.5 mg/ml soybean trypsin inhibitor, 0.1 mg/ml DNase in DME/F-12 and gently shaken for 30 min. The collagenase/dispase treatment removed myoid cells which were washed free of the tubules by allowing the tubules to settle and resuspending in DME/F-12. Fresh collagenase/dispase mixture was then added and the tubules disrupted into small clumps and single cells by gentle pipetting. The resultant enriched Sertoli cell fraction was filtered through a 100-or 111-μm Nitex mesh (Tetko, Inc., Elmsford, NY), and washed twice in DME/F-12 containing 1–5 mg/ml BSA or 10% FCS.

Incubation of Sertoli Cells with STBM Sleeves

Freshly washed Sertoli cell preparations from either mouse or rat testis were suspended in DME/F-12 containing 10% FCS and added immediately to a suspension of STBM sleeves. Typically, 5–10 × 10⁶ cells were added to STBM sleeves derived from one-fourth to one-sixth of the extracellular matrix obtained from a single adult rat testis. Incubation of the Sertoli cells and STBM was conducted at room temperature on a rotary shaker (∿60 cycles/min) for 2–4 h. Polypropylene centrifuge tubes were used to minimize nonspecific adherence of STBM to the reaction vessel. Sertoli cells and STBM sleeves were transferred into plastic petri dishes for visualization and photography using a Zeiss inverted microscope. In some instances co-cultures were agitated for additional time (2 h to overnight). The viability of Sertoli cells at the end of the incubation period was occasionally assessed by the addition of fluorescein diacetate (Sigma Chemical Co.) to the cultures and subsequent examination using epifluorescence. In other experiments Sertoli cells were incubated with STBM sleeves at 33°C and for periods up to 24 h.

To determine the potential role of fibronectin, Sertoli cells freshly suspended in DME/F-12 containing 5 mg/ml BSA instead of 10% FCS were incubated with STBM sleeves suspended in the same medium. To determine the cellular specificity of the adhesion other cell types were also incubated with STBM sleeves. These control cells consisted of a mixed population of adult mouse spermatogenic cells isolated according to Romrell et al. (1976), including predominantly pachytene spermatocytes, round spermatids, condensing spermatids, and spermatogenic residual bodies. Other control cells used included freshly typsinized 3T3 cells and PTK cells. Additionally, the Sertoli cell enriched fraction was treated with trypsin or pronase (0.5 mg/ml at 25°C for 20 min) then washed twice in DME/F-12 plus 10% FCS before incubation with STBM sleeves.

Enzymatic Digestion of STBM Sleeves

STBM sleeves were suspended in the following lytic enzymes each at 1 mg/ml or as indicated in DME/F-12: trypsin (Sigma Chemical Co.), pronase (Calbiochem-Behring Corp.), proteinase K (EM Biochemicals Inc., Elmsford, NY), heparinase (250 U/ml, Sigma Chemical Co.), and chondroitinase ABC (5 U/ml, Sigma Chemical Co.). Hyaluronidase (3,000 U/ml, Sigma Chemical Co.) was used in 0.01 M EDTA, 0.05 M benzamidine HCl (Aldrich Chemical Co., Milwaukee, WI) at pH 5.4. The STBM sleeves were incubated at 37°C with the proteases for 30 min or with the glycosoaminoglycan cleaving enzymes for 30–120 min. The sleeves were then washed once in DME/F-12 followed by two washes in DME/F-12 containing 10% FCS for subsequent incubation with Sertoli cells. Control studies included the addition of soybean trypsin inhibitor (1 mg/ml) to STBM samples treated with trypsin. Additionally, after trypsinization some STBM samples were incubated with medium containing BSA (1–5 mg/ml), BSA plus laminin (10–100 μg/ml), or 10% FCS plus laminin (10–100 μg/ml).

Chemical Alteration of STBM Sleeves

STBM sleeves were treated as follows: heating to 60°, 70°, 80°, or 90°C for

10 min; 0.1 M sodium metaperiodate at 37°C for 30 min; 4 M guanidine hydrochloride at 25°C for 30 min; or 4 M NaCl at 25°C for 30 min. STBM sleeves were washed three times in DME/F-12 after these treatments, before being incubated with the Sertoli cell–enriched fraction.

Ultrastructural Procedures

STBM sleeves or Sertoli cells incubated with STBM sleeves were fixed in 1% paraformaldehyde, 3% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4), postfixed with 1% OsO$_4$ in 0.1 M Na cacodylate buffer and 5% sucrose, en block stained with uranyl acetate, dehydrated in ethanols, and embedded in Epon/Araldite. Thick sections were stained with toluidine blue, while thin sections were stained with uranyl acetate and lead citrate before examination with a Philips 200 transmission electron microscope at 60 kV.

Results

Characteristics of the STBM Sleeves

When examined by dark-field or phase-contrast microscopy the isolated STBM appeared as acellular, lacy, hollow tubes or sleeves which floated in the medium (Fig. 1). While most of the STBM appeared as sleeves, some of the STBM collapsed either partially or completely during their preparation. In addition to the distinctive STBM sleeves, other extracellular matrix material appeared as amorphous masses and as the extracellular matrix of blood vascular elements.
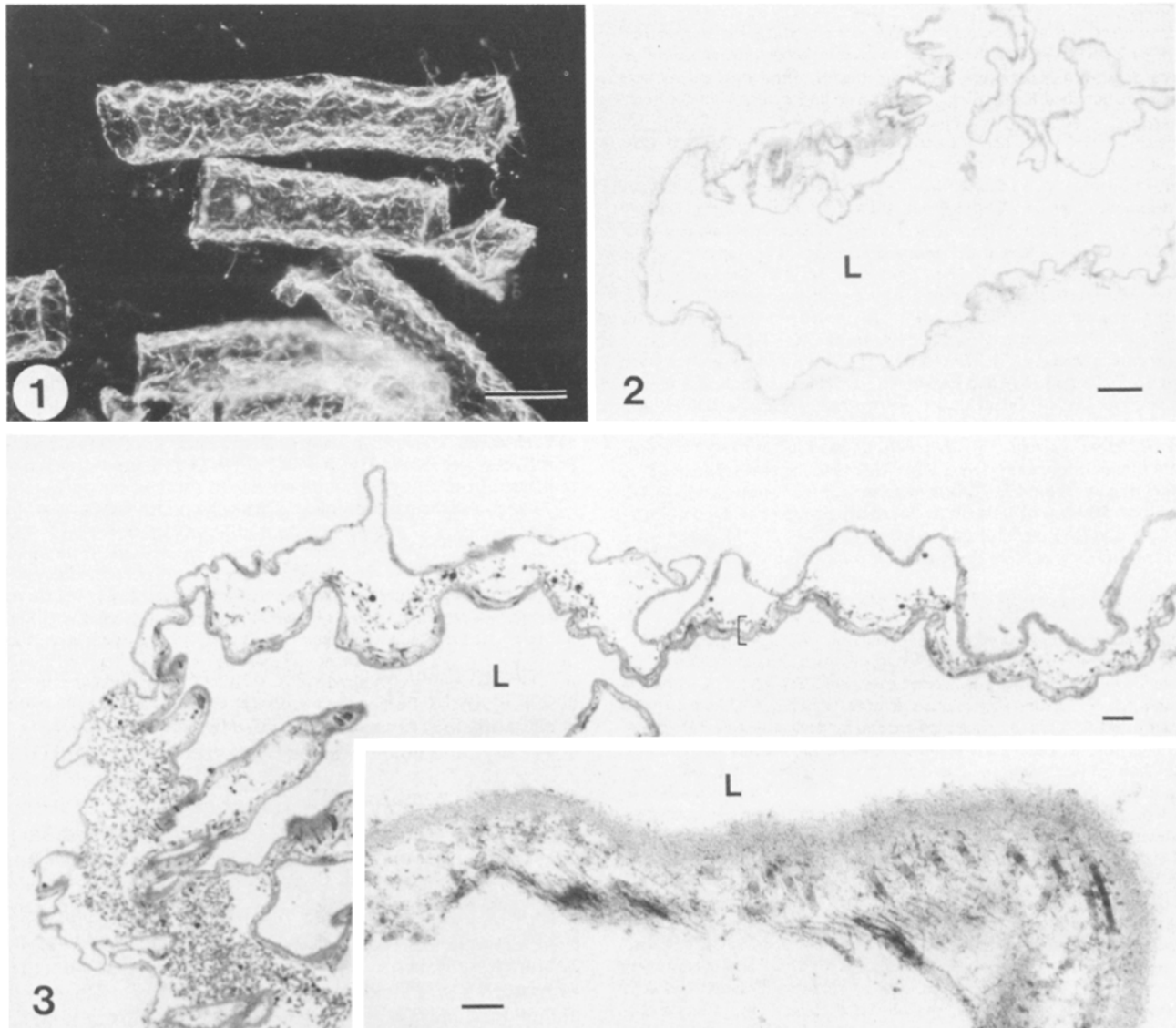
Figures 1–3. (Fig. 1) Dark-field micrograph of rat STBM in the final stage of preparation (in 1% Na deoxycholate, just before rinsing into DME/F-12). The sleeves appear as lacy hollow tubes. Bar, 200 µm. (Fig. 2) Bright-field micrograph of a thick section of rat STBM embedded in Epon/Araldite and stained with toluidine blue. The dual nature of the basement membrane is evident with the thicker, inner layer and thinner, outer layer which separates from the inner layer. L, lumen, where Sertoli cells and spermatogenic cells resided in vivo. Bar, 10 µm. (Fig. 3) Electron micrograph of thin-sectioned STBM. The STBM is composed of two portions: a thicker inner or lumenal basal lamina with associated collagen fibrils (bracket and insert), and an outer, thinner basal lamina which tends to separate away from the inner layer. Bar, 1 µm. The insert represents an area similar to the bracketed region, illustrating the structure of the inner basal lamina. L, lumen of STBM. Bar, 100 nm.

These latter structures could easily be distinguished from the STBM sleeves because the vascular tubes branched, varied greatly in diameter, and had much thicker walls than did the sleeves of seminiferous tubules (compare Figs. 1 and 15).

Thick (Fig. 2) and thin (Fig. 3) sections of the fixed and embedded STBM sleeves confirmed that they were acellular, but revealed that their wall was composed of two major parts: (a) an inner layer consisting of a thick basal lamina and subjacent fibrillar region containing collagen fibrils (seemingly characteristic of type I collagen), and (b) an outer layer consisting of a thinner, occasionally discontinuous basal lamina and sparse fibrillar material. The distance between the two layers varied with the outer layer occasionally appearing to contact the inner layer.

Analysis of the STBM sleeves by SDS PAGE indicated at least 11 major Coomassie Blue–stained bands, ranging from material that could not enter the separation gel to components as small as 44 kD (Fig. 4). Material was present at those molecular weights consistent with expected migration of laminin, fibronectin, and type IV collagen.

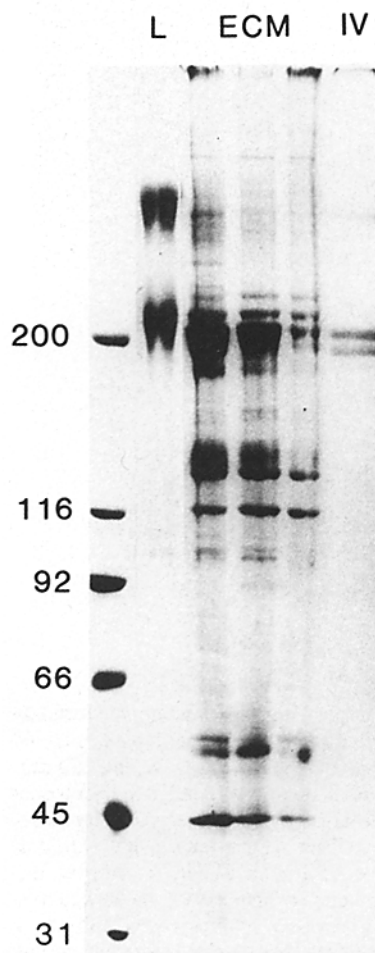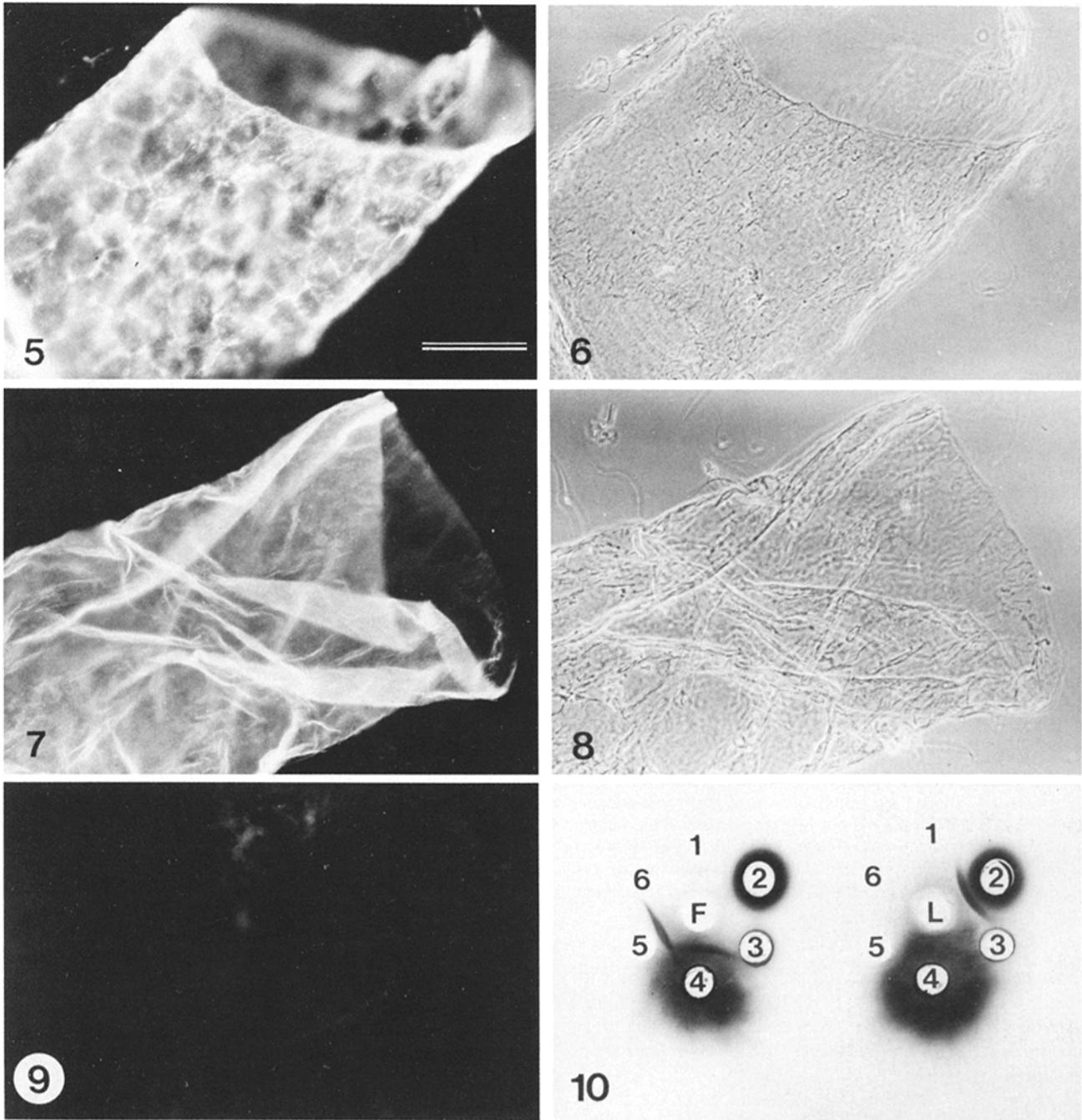The immunofluorescent localization of fibronectin within

Figure 4. Coomassie Blue–stained SDS PAGE of rat STBM on a 4–10% acrylamide gradient gel. The lanes from left to right are protein standards (molecular mass in kilodaltons), laminin (*L*), three different loadings of rat STBM (*ECM*), and type IV collagen (*IV*). Material in STBM includes components that appear to co-migrate with laminin and type IV collagen, in addition to a number of other prominent protein bands.

the STBM sleeves revealed a hexagonal pattern 30–50 μm across (Fig. 5), reminiscent of the outline of cellular boundaries. In some experiments this hexagonal pattern was less apparent and more uniform fluorescence was present. Phase-contrast images of the same STBM sleeves revealed no corresponding hexagonal pattern (Fig. 6). The localization of laminin (Fig. 7) resulted in a more uniform immunofluorescent pattern that appeared to follow each fold of the STBM sleeves (Fig. 8). When nonimmune rabbit sera was used instead of antifibronectin or anti-laminin only light, spotty immunofluorescence was observed (Fig. 9). Addition of 30% FCS or 100 μg/ml of laminin to the primary antisera reduced the fibronectin and laminin immunofluorescence to background levels, respectively. Analysis of the antisera by Ochterlony immunodiffusion revealed that the fibronectin and laminin antisera reacted with the expected antigens and did not cross-react with each other or with type IV collagen (Fig. 10).

Incubation of Cells with STBM Sleeves

Enriched populations of primary Sertoli cells consisted of single cells and small cell aggregates containing both Sertoli cells and early spermatogenic cells. When these Sertoli cell–enriched fractions were incubated with the STBM sleeves and gently shaken for 2–24 h, three types of cellular adhesion occurred: cells bound to other cells; cells bound to the petri dish; and cells bound to the STBM sleeves. Those Sertoli cells that bound to the STBM sleeves did so asymmetrically with cells adhering predominantly to the ends of the STBM tubes (Fig. 11). As the length of incubation time increased, all types of cell adhesion increased, including cell–STBM sleeve adhesion. Cell attachment was generally greatest to those STBM sleeves that retained their hollow configuration, allowing cells access to their inner surface at the ends of the sleeves. Some cell binding did, however, occur at the ends of STBM sleeves that were partially collapsed. Occasional STBM sleeves had nicks along their length. Cell binding occurred at these sites (Fig. 12). Starting Sertoli cell–enriched fractions were generally 85% viable. After incubation with STBM sleeves for 2–6 h, staining with the vital dye fluorescein diacetate confirmed that the cells adhering to the STBM sleeves remained viable, and that some of the nonadhering cells were not (Figs. 13 and 14).

The asymmetric cell binding was specific for the STBM sleeves since the contaminating segments of vascular extracellular matrix bound only occasional cells of the Sertoli cell–enriched fraction (Fig. 15). Cells were often seen binding to portions of the amorphous masses of extracellular matrix material, but in a seemingly random manner. Although most experiments were performed with Sertoli cell–enriched fractions isolated from mice, rat Sertoli cells also bound asymmetrically (Fig. 12). Sertoli cells adhered asymmetrically to STBM sleeves in the presence of 10% FCS or 1–5 mg/ml BSA. In control experiments, when mixed populations of spermatogenic cells isolated from adult mice were incubated with the STBM sleeves little cell binding was seen. The late spermatogenic cells did not adhere to each other, to the STBM sleeves, or to the surface of the petri dishes. However, the incubation of trypsinized 3T3 cells with the STBM sleeves resulted in the binding of these cells uniformly over all surfaces of the STBM sleeves (Fig. 16). PTK cells also seemed to be bound randomly to all surfaces of the STBM.

Figures 5–10. (Fig. 5) Immunofluorescent micrograph of fibronectin immunoreactivity as revealed by indirect immunocytochemical localization of anti–fibronectin antibodies. The hexagonal pattern of fluorescence, 30–50 μm across, probably represents the positioning of myoid or sinusoid endothelial cells in vivo rather than the much small basal laminal contact area of Sertoli cells or spermatogonia. Bar, 100 μm. (Fig. 6) Phase-contrast micrograph of same STBM as Fig. 5. There are no phase-contrast structures evident that correspond to the hexagonal immunofluorescence pattern. Same magnification as Fig. 5. (Fig. 7) Immunofluorescent micrograph of laminin immunoreactivity as revealed by indirect immunocytochemical localization of anti–laminin antibodies. Laminin immunoreactivity appears much more uniform than fibronectin localization. Same magnification as Fig. 5. (Fig. 8) Phase-contrast micrograph of same field as Fig. 7. (Fig. 9) Immunofluorescent micrograph of nonimmune rabbit sera immunoreactivity as revealed by indirect immunocytochemical localization. There is some nonspecific binding to the STBM. Same magnification as Fig. 5. (Fig. 10) Immunodiffusion plate assaying reactivity of anti–fibronectin (*F*) and anti–laminin (*L*) antisera against laminin (well *2*) type IV collagen (well *3*), rat sera (well *4*), fibronectin (well *5*), and rat STBM (wells *6* and *1*). The fibronectin antisera cross-reacted with both fibronectin and rat sera which contains fibronectin. Laminin antisera only reacted with laminin. Fibronectin and laminin within the STBM is not soluble and thus did not form precipitation arcs with the antisera.
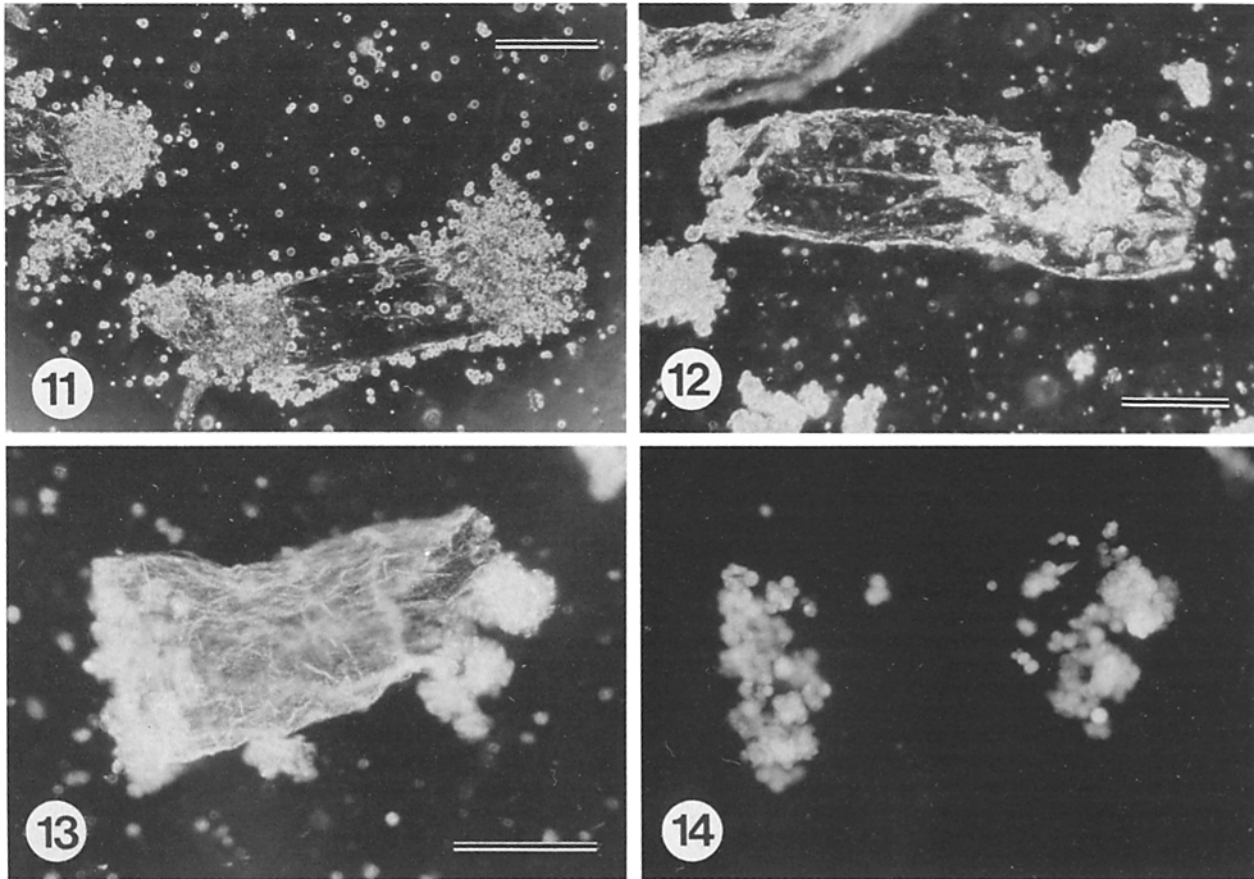
Figures 11–14. (Fig. 11) Dark-field micrograph of STBM sleeves incubated with enriched mouse Sertoli cells overnight at room temperature. Note the binding of cells to the cut ends of the STBM and to each other. Bar, 200 μm. (Fig. 12) Dark-field micrograph of STBM sleeves incubated with enriched rat Sertoli cells for 4 h at room temperature. Cells bind to the end of the STBM sleeve and also in the region where the STBM has been nicked, allowing access to the lumenal surface. Bar, 200 μm. (Figs. 13 and 14) Dark-field micrograph (Fig. 13) of enriched mouse Sertoli cells incubated with STBM for ~4 h at room temperature before labeling with fluorescence diacetate. Fluorescent micrograph (Fig. 14) of same STBM sleeve showing live cells at the ends of the STBM also indicates that some of the nonadherent cells and cells not binding to the STBM ends do not concentrate fluorescence diacetate and are not viable. Bar, 200 μm. Figs. 13 and 14 are same magnification.

When the Sertoli cell–enriched fraction was treated with trypsin and then incubated with the STBM sleeves, cells still adhered asymmetrically, but fewer cells bound at any given time compared with untreated cell fractions. Pronase treatment inhibited essentially all cell binding.

Thick (Fig. 17) and thin (Fig. 18) sections of the Sertoli cell–enriched fraction shaken with STBM sleeves for 24 h at 33°C confirmed that the cells bound to the STBM sleeves were Sertoli cells to which a few early spermatogenic cells still adhered. The sections confirmed that the Sertoli cells bound predominantly to the inner or lumenal surface of the STBM sleeves, their in vivo location. The Sertoli cells adhered closely to the highly folded thick, inner basal lamina on its lumenal surface when incubated together at 33°C for 24 h. Occasionally Sertoli cells were seen adhering to the outer, thin basal lamina but their plasma membranes did not follow the contours of the outer basal lamina, and made only focal contacts.

Effects of Enzymatic Digestion of the STBM Sleeves

Treatment of the STBM sleeves with trypsin only slightly altered their phase-contrast or dark-field microscopic appearance. The STBM sleeves seemed slightly larger in diameter, but they still retained their lacy, tubular form. The amount of extracellular matrix material that appeared as amorphous masses increased, but the blood vascular extracellular matrix material appeared unchanged. Although both pronase and proteinase K treatment dramatically reduced the number of identifiable STBM sleeves, some tubular forms were present after either treatment. Neither chondroitinase ABC, heparinase, nor hyaluronidase affected the phase-contrast or dark-field appearance of the STBM sleeves.

A series of studies were also performed to examine the effects of proteolytic treatment on the adherence of Sertoli cells to the STBM sleeves. STBM tubules pretreated with trypsin, pronase, or proteinase K did not bind Sertoli cells. The addition of soybean trypsin inhibitor during trypsinization of the sleeves allowed subsequent asymmetric Sertoli cell binding. Pretreatment of the STBM sleeves with chondroitinase ABC (Fig. 21), heparinase, or hyaluronidase did not inhibit asymmetric Sertoli cell adhesion. The addition of soluble laminin (10–100 μg/ml) during incubation of the Sertoli cell–enriched fraction with STBM sleeves pretreated with trypsin restored some cell–STBM sleeve binding, but binding seemed random. Clumps of cells bound to all sur-

faces of the STBM sleeves (Fig. 22), but never completely covered the external surface of the sleeves.

Effects of Chemical Alteration of STBM Sleeves

Heating STBM sleeves to 60–90°C did not alter their light microscopic appearance, nor did heating to 60°–70°C inhibit the asymmetric adhesion of Sertoli cells to the STBM tubes. However, heating of the STBM to 80°–90°C reduced the asymmetric binding of Sertoli cells to the extracellular matrix material. Asymmetric Sertoli cell–STBM binding was also inhibited by treatment of the STBM sleeves with sodium metaperiodate. Treatment of the STBM with 4 M NaCl did not inhibit subsequent asymmetric adhesion of Sertoli cells. In contrast, 4 M guanidine HCl completely prevented Sertoli cell–STBM sleeve binding. Guanidine HCl and sodium metaperiodate pretreatment of STBM sleeves did not, however, prevent the self-aggregation of Sertoli cells or their adhesion to the petri dish surface.

Discussion

The STBM sleeve isolation procedure (Reddy et al., 1983, Watanabe et al., 1984) is mild and nonenzymatic, other than the addition of DNase. The isolated STBM sleeves retain the histotypic form of the seminiferous tubules, both their three-dimensional structure as hollow tubes, and in their unique ultrastructural appearance. As described by Hermo and Clermont (1976) the limiting membrane of the rat seminiferous tubule in vivo consists of two extracellular components: (a) the inner lamella composed of a basal lamina adjacent to the Sertoli and spermatogenic cells with collagen fibrils embedded in amorphous material next to the myoid cells; and (b) the outer lamella composed of a thin second basal lamina in which a few collagen fibrils are embedded. The outer lamella lies between the peritubular myoid cells and the endothelial cells lining the intertubular lymphatic sinusoids (see Fig. 5, Dym and Fawcett, 1971). The isolated STBM sleeves retained this dual nature with a thick lumenal basal lamina with adjacent collagen fibrils and a less prominent outer layer retaining a few collagen fibrils and a thinner basal lamina. Within the fibrillar region of the thick inner layer were small patches of basal lamina–like material. Fig. 23 is a schematic diagram of our interpretation of the isolated STBM, showing its relationship to the cellular components of the seminiferous tubules in vivo. The dual nature of our isolated STBM sleeves is consistent with the work of Watanabe et al. (1984, see Figs. 2 and 10), although the presence of two basal laminae was not discussed in their report.

In addition to retaining their histotypic form, the isolated STBM sleeves appear to retain those biochemical components known to be present in vivo. Tung et al. (1984) have demonstrated that antigens that cross-react with a monoclonal antibody to fibronectin are present in a peritubular manner within cryostat sections of 20-d-old rat testes. They have also shown that fibronectin synthesis may be used as a marker for peritubular myoid cells in vitro. Tung and Fritz (1984) have also identified immunocytochemically fibronectin, laminin, and type IV collagen within an extracellular matrix preparation from adult rat testes that, however, involved homogenization of their preparation. Skinner et al. (1985) have further reported that isolated rat peritubular myoid cells in culture produce fibronectin, and types I and IV
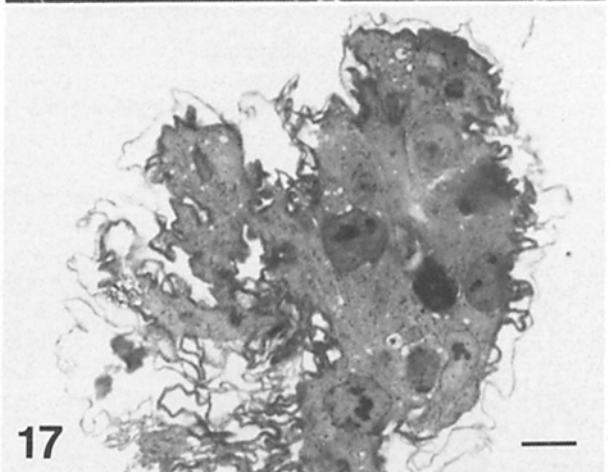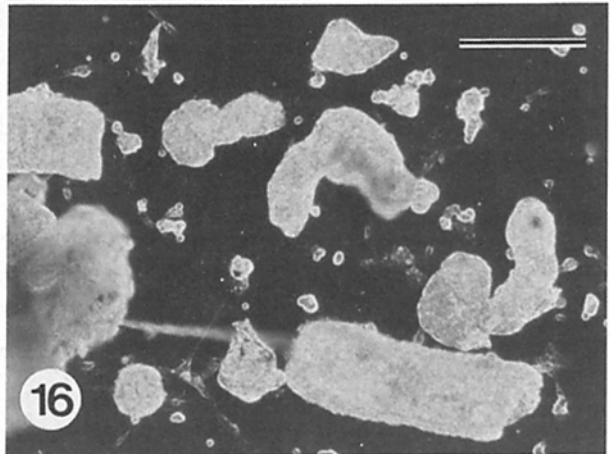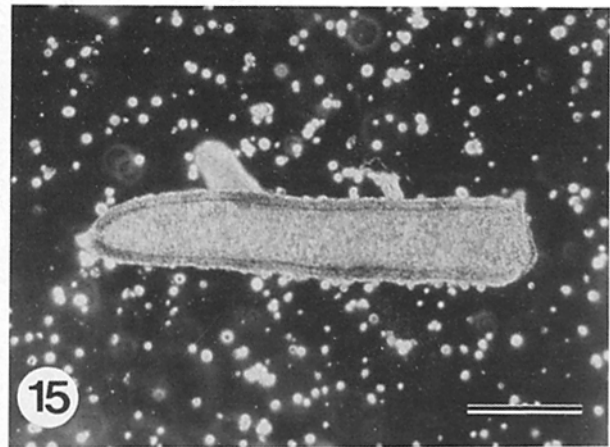
Figures 15–17. Dark-field micrograph of the extracellular matrix of a blood vessel contaminating the STBM preparation. This preparation had been incubated with enriched Sertoli cells at room temperature for ~4 h. Only occasional cells adhere to the vascular extracellular matrix. Bar, 200 μm. (Fig. 16) Dark-field micrograph of STBM sleeves incubated with 3T3 cells overnight at room temperature. Note that the 3T3 cells bind to all available surfaces of the STBM sleeves. Bar, 200 μm. (Fig. 17) Bright-field micrograph of a toluidine blue–stained thick section across the end of a STBM sleeve that had been incubated with mouse Sertoli cells for 24 h at 33°C. The Sertoli cells generally adhere closely to the thicker inner basal lamina, while the thinner, outer basal lamina often separates from the inner layer. Bar, 10 μm.

Figure 18. Transmission electron micrograph of a thin section of the same material described in Fig. 17. The Sertoli cell has formed an extensive region of contact with the inner basal lamina. Note the abundant adjacent collagen fibrils and small segments of additional basal lamina-like material. The thinner, outer basal lamina has separated from the inner layer. Bar, 1 μm.

collagens, whereas Sertoli cells produce type IV collagen and laminin. When myoid and Sertoli cells were co-cultured, fibronectin, laminin, and types I and IV collagen were secreted extracellularly. This in vitro work is consistent with the work of Hadley et al. (1985) who have used immunocytochemistry to localize laminin and type IV collagen within the inner basal lamina adjacent to the Sertoli cells and fibronectin and type I collagen in a more diffuse pattern within the interstitium of the human testis. Based on SDS PAGE and immunocytochemistry, our STBM preparation contains laminin, fibronectin, and putative type IV collagen. Ultrastructural images suggest that type I collagen may also be present. The hexagonal immunofluorescent pattern (30–50 μm across) obtained when fibronectin was localized in the STBM is consistent with fibronectin deposits at the edges of flattened peritubular myoid or sinusoidal endothelial cells, rather than the much smaller bases of Sertoli cells or spermatogonia (15–20 μm).

The STBM sleeves also retain biological activity in that Sertoli cells perferentially adhere to the lumenal surface of the sleeves, their in vivo location. When initially observed, we were concerned that the asymmetric binding might be due to reduced shear forces cells experience inside the tubes as opposed to those on the outer surface. However, this does not

appear to be responsible for the lumenal Sertoli cell binding since Sertoli cells do not bind to the inner surface of the extracellular matrix casts of large blood vessels that have diameters similar to the STBM sleeves (see Fig. 15). The binding of Sertoli cells to nicked regions of the STBM sleeves also indicates that there is nothing special about the ends of the STBM sleeves; simple access to the inner surface of the sleeves is all that is necessary. Short (~400 μm) segments of STBM were often completely covered with adherent Sertoli cells on their inner surface. The asymmetric cell binding is probably not due to the exposure of "sticky" extracellular matrix material at the edges of the sleeves since spermatogenic cells did not bind to the cut ends. The asymmetric adhesion of the Sertoli-enriched fraction to the STBM is also probably not due to the retention of basement membrane components on the Sertoli cell fraction after collagenase/dispase treatment since trypsinized Sertoli cells still bound asymmetrically (data not illustrated).

The binding of both 3T3 cells and PTK cells, seemingly random and nonasymmetrical all over the STBM sleeves, indicates that the external surface can bind certain cells. These results are consistent with the results of Watanabe et al. (1984) who reported that pancreatic acinar carcinoma cells bound all over STBM sleeves when incubated together for
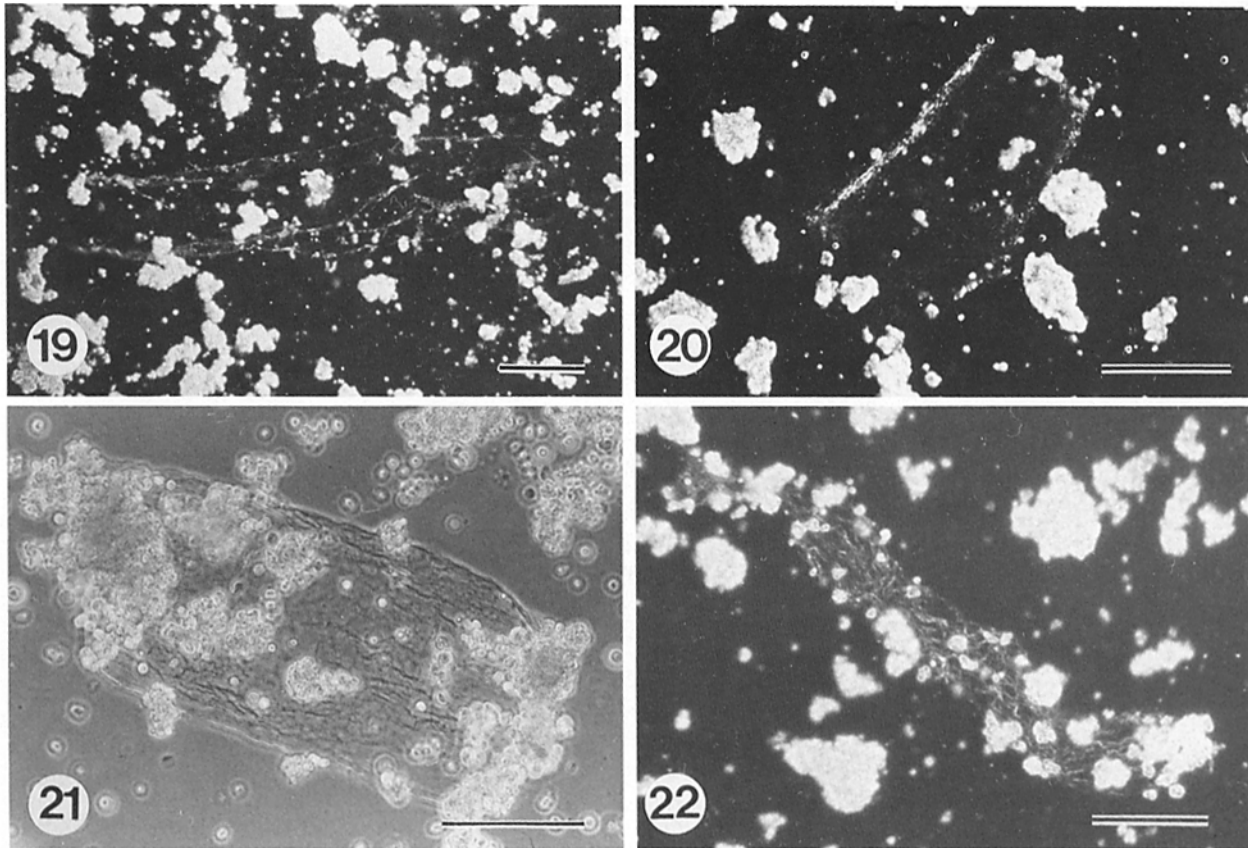
Figures 19–22. (Figs. 19–20) Dark-field micrograph of STBM sleeves that had been pretreated with trypsin (Fig. 19) or pronase (Fig. 20), and then incubated with enriched Sertoli cells for ~4 h at room temperature. While cell–cell binding still occurred, few cells bound to the proteinase-treated STBM sleeves. Bar, 200 μm. (Fig. 21) Phase-contrast micrograph of a STBM sleeve that had been pretreated with chondroitinase ABC and then incubated with enriched Sertoli cells overnight at room temperature. Asymmetric Sertoli cell binding still occurred. Bar, 200 μm. (Fig. 22) Dark-field micrograph of a trypsin-pretreated STBM sleeve that had been incubated with enriched Sertoli cells in the presence of soluble laminin for ~4 h at room temperature. Sertoli cells bound at the ends and in clumps along the outer surface of the STBM sleeves. Bar, 200 μm.

1–6 h. These cells are probably adhering to the thin, outer basal lamina formed between the peritubular myoid cells and the lymphatic endothelial cells.

Since Sertoli cells bind preferentially to the inner, lumenal basal lamina of the STBM sleeves, there are probably components recognized by Sertoli cells present in this basal lamina. Asymmetric Sertoli cell binding was inhibited by a variety of proteases, suggesting that proteins, glycoproteins, or proteoglycans are potential cell-binding components. The inhibitory effect of trypsinizing the STBM sleeves was probably not due to residual protease activity present since cell–cell and cell–petri dish adhesion was not inhibited. Within a number of other cell-binding systems, glycosaminoglycans have been implicated in cell adhesion (Toole, 1981; Cole and Glaser, 1984; Turley, 1984). Under the conditions we used, neither chondroitinase ABC which cleaves hyaluronate, chondroitin, chondroitin sulfate, and dermatin sulfate, heparinase which cleaves heparin and heparan sulfate, nor hyaluronidase were effective inhibitors of Sertoli cell–STBM adhesion. Yet 4 M guanidine HCl, which is often used to extract proteoglycans (Breton et al., 1985), did inhibit Sertoli cell–STBM binding. Carbohydrate may be involved since the cell binding was sensitive to Na metaperiodate treatment.

Fibronectin does not appear to be involved in the Sertoli cell–STBM interaction, since the presence or absence of soluble fibronectin had no influence on asymmetric Sertoli cell binding. This is consistent with the work of Tung and Fritz (1984) who reported that Sertoli cells bound to a homogenized extracellular matrix preparation of rat testes maintained in serum-free, chemically defined media.

Our results suggest that laminin may be one of several components of the STBM sleeves to which Sertoli cells bind. Previous workers have shown that the cell binding activity of laminin is relatively resistant to thermal denaturation (Johansson et al., 1981; Fridman et al., 1985), but sensitive to sodium metaperiodate treatment (Johansson et al., 1981; Aplin et al., 1984; Fridman et al., 1985). On the other hand, the cell binding activity of fibronectin is sensitive to thermal denaturation (heating to 70°C), but resistant to sodium metaperiodate treatment (Johansson et al., 1981; Fridman et al., 1985). Linsenmayer et al. (1984) have also reported that an epitope of type IV collagen within a variety of different basement membranes is denatured when heated to 50°–55°C. We found that heating the STBM sleeves to 60°–70°C had little effect on asymmetric Sertoli cell adhesion, but that heating to 80°–90°C or treatment with sodium metaperiodate inhibited Sertoli cell binding.
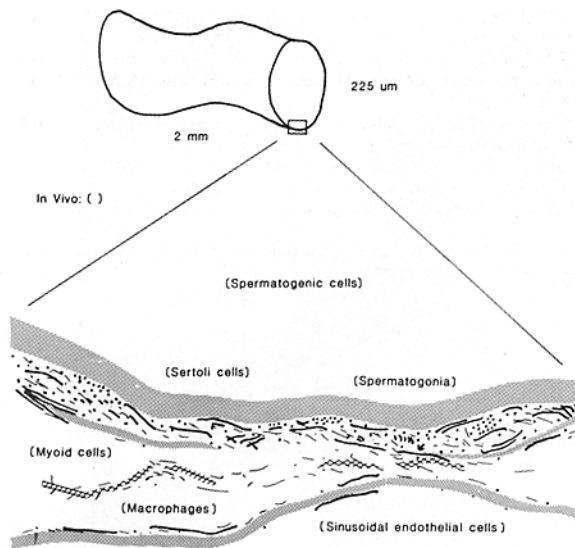
Figure 23. Schematic diagram illustrating the ultrastructural relationships of the isolated STBM sleeves to the in vivo cell types. Note the isolated STBM consist of two layers: An inner layer containing a thick lumenal basal lamina (upon which Sertoli cells and spermatogonia rest in vivo) with subjacent collagen fibrils, and an outer layer consisting of a thin basal lamina, with occasional collagen fibrils. The outer layer was produced in vivo between peritubular myoid cells and sinusoidal endothelial cells.

However, asymmetric distribution of laminin with STBM alone cannot explain our results. When soluble laminin was added to the incubated Sertoli cells and STBM sleeves in 1–5 mg/ml BSA or 10% FCS, the presence of laminin did not inhibit asymmetric binding; if there was any influence it was to stimulate cell binding to the STBM sleeves in general. The most dramatic effect of soluble laminin occurred during incubation of Sertoli cells with STBM sleeves that had been pretreated with trypsin. The addition of soluble laminin allowed consistent, but seemingly random binding of Sertoli cell aggregates to the external surface of the treated STBM sleeves. The presence of 10% FCS had no influence on this process. When the STBM sleeves were incubated in either anti–fibronectin or anti–laminin polyclonal antisera, then mixed with Sertoli cells, no inhibition of the asymmetric binding was observed (Enders, G. C., unpublished observations). Fridman et al. (1985) found similar results when they preincubated subendothelial cell extracellular matrix with anti–laminin antibodies before the addition of colon carcinoma cells, which are thought to display laminin-mediated binding.

Our results lead us to suggest that Sertoli cells probably have receptors for laminin, but that there are additional extracellular matrix components, asymmetrically distributed in the STBM sleeves to which Sertoli cells can bind.

Our in vitro cell–extracellular matrix model system allows us to examine cell binding to two different in vivo produced basal lamina simultaneously: one produced between Sertoli/spermatogonia cells and myoid cells; and the other between myoid cells and sinusoidal endothelial cells. The results indicate that Sertoli cells recognize differences between these two basal laminae. Preliminary experiments indicate that primitive type A spermatogonia display similar binding preference for the lumenal basal lamina as Sertoli

cells (Enders, G. C., unpublished observations). It is also interesting to note that in vivo macrophages migrate through the outer basal lamina but do not normally cross the inner basal lamina. This may be another indication of molecular differences between the two basal laminae.

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