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Characterization of a New Superfusion, Two-Compartment Culture System for Sertoli Cells: Influence of Extracellular Matrix on the Cell Permeability and Dynamics of Transferrin Secretion

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A new superfusion two-compartment culture system was developed in the laboratory of the author and was used to investigate the influence of testicular extracellular matrix on barrier formation by Sertoli cells in culture, and the acute dynamic changes in the bidirectional secretion of transferrin. Only Sertoli cells growing on extracellular matrix formed a monolayer that was specifically impermeable to inulin, peroxidase, and FSH, but did not affect the passage of testosterone. Moreover, in these conditions, they were highly polarized morphologically. The bidirectional secretion (basal/apical ratio) of transferrin was affected by the duration of the stationary culture preceding the superfusion. After 2 days of culture, the amount of transferrin secreted during the subsequent 20 h superfusion was higher in the basal chamber than in the apical chamber. In contrast, after 5 days of culture, the amount of secreted transferrin was higher in the apical compartment. The author compared his data with those previously reported by other authors, using stationary two-compartment chambers.

Key words: two compartment culture system, sertoli cells, extracellular matrix, superfusion, transferrin, rat.

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The seminiferous epithelium of the adult testis is composed of developing germ cells intimately associated with Sertoli cells which are involved in the maintenance and regulation of spermatogenesis. Maturation of the later germ cell types is dependent upon the creation of a specialized environment by Sertoli cells. This specialized environment is separated from the systemic circulation by Sertoli cell tight junctions which reside in the basal region of the cell and institute the morphological basis of the blood testis barrier (Dym and Fawcett, 1970). Since the tight junctional complexes prevent any free access of components from blood plasma to germ cells in the adluminal compartment, the Sertoli cells are able to regulate the environment in which the latter germ cells divide and differentiate (Dym, 1977). Indeed, the composition of seminiferous tubular fluid is quite different from the composition of serum (Setchell and Brooks, 1988). The production of this unique environment is at least partly due to the secretion of proteins by Sertoli cells. These proteins include transferrin (Skinner and Griswold, 1980), androgen binding protein (Fritz et al, 1976), ceruloplasmin (Skinner and Griswold, 1983), plasminogen activator (Lacroix et al, 1977), and

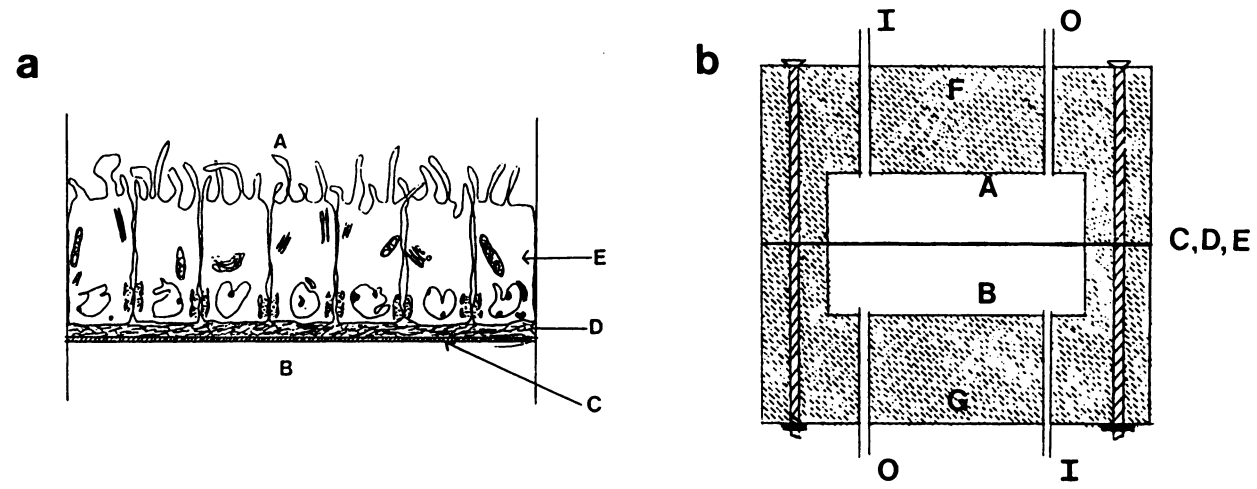

Fig. 1. Schematic representation of the superfusion two-compartment culture chamber (magnification 2). View a: cross section of the culture chamber. (C) = millipore filter, (D) = testicular extracellular matrix, (E) = Sertoli cells monolayer. View b: (A) = apical compartment, (B) = basal compartment, (I - O) = medium inlets and outlets, (F) = top plexiglass cylinder, (G) = bottom plexiglass cylinder.

clusterin (Blaschuk et al, 1983). Sertoli cells have been successfully cultured *in vitro* in a number of laboratories for over 10 yr, and this has provided a large amount of information concerning hormonal regulation and secretory activity (Mather et al, 1983). However, the commonly used standard culture conditions provide a highly artificial environment, especially for functionally differentiated epithelial cells. This is reflected by flat cell appearance, loss of ultrastructural and functional polarity, and relatively fast disappearance of differentiated functions (Hadley et al, 1985). For the last few years, attempts have been made to improve Sertoli cells morphology and extend their secretory activity in culture. Indeed, the phenotypic structure and function of Sertoli cells can be maintained to a large degree *in vitro* by growing cells on a reconstituted basement membrane extracellular matrix (Byers et al, 1986). More recently, culture chambers have been developed (Djakiew et al, 1986; Janecki and Steinberger, 1986) in which Sertoli cells are grown at high density on a permeable support with or without an appropriate extracellular matrix. In such chambers, they establish confluent epithelial sheets of highly differentiated, morphologically polarized cells and they secreted transferrin, androgen binding protein, plasminogen activator, and inhibin bidirectionally (Ailenberg and Fritz, 1988; Janecki and Steinberger, 1987; Hadley et al, 1987; Ueda et al, 1988; Handelsman et al, 1989). Nevertheless, static cultures do not permit continuous measurement of the dynamic changes in the secretory pattern or in hormone receptor interactions. In the present paper, a new culture system is described which combines the advantages of a two-compartment culture chamber with those of a continuous superfusion to investigate the acute dynamic changes in the secretory pattern and hormonal controls. The paper provides a characterization of Sertoli cells monolayers maintained in such a two-compartment culture system, emphasizes the importance of extracellular matrix on monolayer permeability, and describes the effects of the culture duration and of medium supplementation on the bidirectional secretion of transferrin.

Materials and Methods

Two-Compartment Superfusion Culture Chamber

The chamber was designed and constructed in this laboratory (Figs. 1a, and b). It consists of an apical (A) and a basal (B) compartment, separated by a permeable membrane (C) (Millipore filter, HA, 0.45 μm) which supports the extracellular matrix (D) and the cell monolayer (E). The top (F) and bottom (G) walls of the chamber are formed by a plexiglass cylinder with external dimensions of 30 mm in diameter and 2 × 12.5 mm in height, and internal dimensions of 18 mm in diameter and 2 × 5 mm in height. The inlets (I) and outlets (O) in each compartment were made of stainless steel tubing (ϕ 1.2 mm) sealed into the plexiglass cylinder. After a stationary culture phase, the Millipore filter is put down onto the bottom plexiglass cylinder (G) and the top plexiglass cylinder (F) is pressed upon the Millipore filter

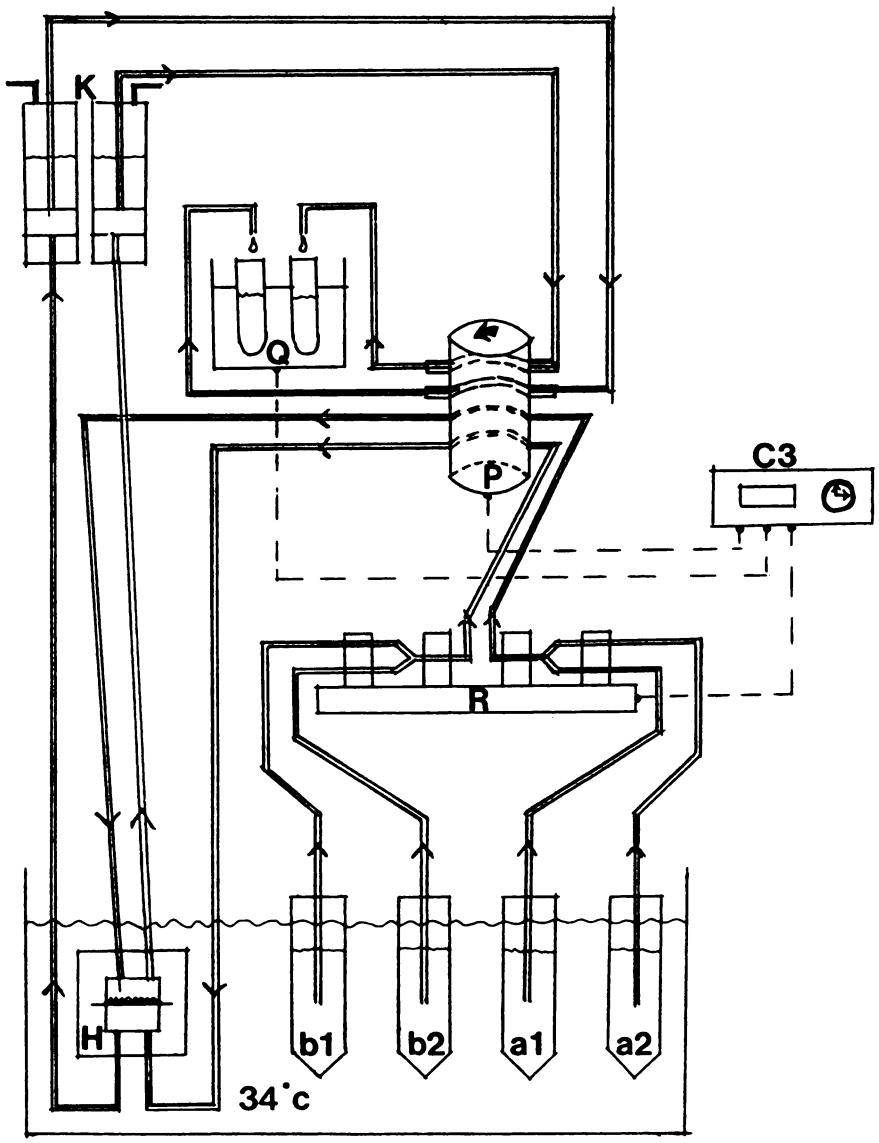

Fig. 2. Schematic diagram of the entire superfusion system. The system and its components are described in the materials and methods section. The diagram does not reflect the actual proportion. (a1, a2) and (b1, b2) = medium reservoirs, (P) = peristaltic pumps, (R) = electromagnetic valve system, (C3) = controler, (K) = collecting unit, (Q) = fraction collector, (H) = culture chamber.

(C) and held firmly by three screws. The chambers can then be perifused.

Two-Compartment Superfusion Culture System

A schematic diagram of the superfusion system is shown (Fig. 2). Only one chamber (H) was used at a time. Tanks (a1, a2 for Apical chamber and b1, b2 for basal chamber) contain sterile culture media. Medium from the tanks is drawn by a peristaltic pump (P) (four-channel minipuls two, Gilson Medical) through an electro-vanne system (R) and enters the chamber compartments (H). The effluents accumulate in a vertical collecting unit (K) constructed with a drop perfusion system. Inside the collecting unit, polyethylene tubing is inserted and connected to a peristaltic pump (P) which drives the medium into the fraction collector (Q) (Gilson, Medical Electronics, France). The electromagnetic valve system is equipped with two three-way electromagnetic valves for buffer delivery from several different media tanks into the apical and basal compartments, and two slow-stop electromagnetic valves for tightness. All connections within the system are made of polyethylene tubing (ID 1.14 mm, OD 1.57 mm; Biotrol Pharma, France), except for the electromagnetic valve system (Silastic Medical grade tubing, Dow Corning; ID 0.62 mm, OD 1.25 mm) and pump (Gilson tubing; ϕ = 0.76 mm for chamber perfusion; ϕ = 1 mm for collecting fraction). The pump, the electromagnetic valves, and the fraction collector are operated by a process controler (C3, Pharmacia, France). Thus, hormones or other test substances can be delivered in a programmed manner. During the experiment, the culture chamber and mediums are kept inside a 34 C water bath; all other equipment remains at room temperature.

Sertoli Cell Isolation

Sertoli cells were isolated from 17–18-day-old rat testes (Wistar, INRA 03), using the method previously described (Guillou et al, 1986). The Sertoli cell preparation was contaminated with 17,5 ± 4.6% germ cells and 4 ± 2% myoid cells. The numeration of cell preparation for germ cells was achieved by staining with trioxy hematein ferric and for myoid cells by phosphatase alcalin reaction (Chapin et al, 1986).

Leydig Cell Isolation

Leydig cells were isolated from 52-day-old rat testes (Wistar, INRA 03), using the method previously described (Guillou et al, 1985). Briefly, the testes were removed and carefully decapsulated. Cells were dispersed by a collagenase digestion (0.25 mg/ml L 15 medium (Serva); 2 ml/testis) for 10 min at 34 C under continuous shaking. After settling of the tubular tissue in a glass cylinder, the supernatant was recovered and centrifuged for 5 min at 80 g. The cell pellet was resuspended in 2 ml L 15 medium and put onto a Percoll discontinuous gradient (Pharmacia 17, 42, and 70%). After centrifugation (80 g, 30 min), the Leydig cells were recovered from the 42% Percoll phase and washed twice in L 15 medium.

Cell Culture

The chamber was sterilized by UV light for 15 min. All solutions used for washing the culture chamber were sterilized by passage through Millex GS 0.22 μm pore size filter units (Millipore). The culture chambers were washed for 2 h in distilled water. Subsequently, the Millipore filter was washed again in distilled water for 24 h and was placed in Petri dishes containing Dulbecco's Minimum Essential Medium (DMEM, Gibco, France) for 1 h. Sertoli and Leydig cells were grown at a density of 1.5×10^6 cells/cm² on the Millipore filter (uncoated or coated with extracellular matrix), in Petri dishes with DMEM supplemented with 100 U/ml penicillin, 0.25 g/ml fungizone, 100 g/ml streptomycin, 2 g/ml insulin, 5 g/ml human transferrin, 50 ng/ml vitamin A, 200 ng/ml vitamin E, 10^{-9} M hydrocortisone, 10^{-7} M testosterone, 10^{-8}M estradiol, 2 mM glutamine, 5 ng/ml sodium selenate, 1 mM sodium pyruvate, 22 mM sodium lactate, and 3 g/ml cytosine arabinoside (all from Sigma Chemical Co., France).

Testicular extracellular matrix was prepared from adult Wistar rat decapsulated testes. Seminiferous tubule segments were separated from interstitial elements and extracted with triton × 100 in an hypotonic buffer in the presence of a protease inhibitor (Tung and Fritz, 1984). Millipore filters were coated with μ 210 g/cm² testicular extracellular matrix.

The cell cultures were incubated for 2 or 5 days at 34 C in a humidified atmosphere of 95% air, 5% CO_2. After this stationary culture phase, the permeable membrane was placed into the culture chamber. The assembled chamber was mounted vertically in the superfusion system. Before the experiment, the entire system was equilibrated for 2 h at a flow rate of 1.7–9 ml ± 0.2 ml/h in each compartment. The cultures were superfused for 6 h for permeability study and 16–20 h for transferrin secretion with one of the following two media: (1) Basic medium = L 15 medium supplemented with 100 U/ml penicillin, 0.25 g/ml fungizone, 100 g/ml streptomycin, 2 g/ml insulin, 5 g/ml human transferrin, 200 ng/ml vitamin E, 2 mM glutamine, 5 ng/ml sodium selenate, 1 mM sodium pyruvate, 22 mM sodium lactate, and 3 g/ml cytosine arabinoside; and (2) Basic medium plus 3F = Basic medium supplemented with oFSH CY1115 200 ng/ml, retinol 1,000 ng/ml, and methyl isobutyl xanthine (MIX) 10^{-6} M. The superfusates were collected and stored at −20 C until assayed.

Morphology

For light microscopy, cells were fixed on the Millipore filter in 4% paraformaldehyde and stained with cresyl violet. The preparations were then dehydrated in ethanol and rinsed with toluene to render the filter transparent.

For electron microscopy, the monolayer was fixed in 3% glutaraldehyde, postfixed in ferrocyanide-reduced osmium tetroxide, and embedded in epon. Thin sections were stained with uranyl acetate and examined with a Philips (CM 10) electron microscope. Four cultures on a filter and four cultures on a filter covered with extracellular matrix were examined through a significant number of sections.

Monolayer Permeability

The monolayer permeability to inulin, peroxidase, FSH, and testosterone was determined over a period of 6 or 20 h by superfusing the different molecules into the basal compartment. The epithelial sheet of Sertoli cells on the filter that was used for permeability studies was derived from 2–5 days stationary culture. In additon, a culture chamber containing only the filter, the filter plus Leydig cells, the filter impregnated only with extracellular matrix, or the filter impregnated with extracellular matrix and covered with Sertoli cells or Leydig cells was also examined for their permeability.

To measure the monolayer permeability in the superfused system, 0.8 μg/ml testosterone, 0.2 μg/ml peroxidase (Sigma), 2.5 mg/ml inulin (Tebu), and 0.24 μg/ml crude pFSH were added to the basic medium superfusing the basal compartment. A cell-free culture chamber served as control. Medium samples were collected from the apical and basal compartments. Peroxidase and inulin were measured by colorimetric assay; FSH and testosterone were measured by radioimmunoassay (see below). The results were calculated as percent of the total substance perfused in the basal compartment that was recovered in the basal and the apical compartments, respectively.

Radioimmunoassays of Transferrin, FSH and Testosterone

Transferrin was measured by radioimmunoassay (RIA) according to Le Magueresse et al (1988). Rat transferrin was purchased from Sigma. The usable range of the assay was 0.2–50 ng/tube, with an intraassay coefficient of variation of 8% for samples within a 20–70% binding range. Samples were assayed in triplicate. Culture medium

samples from one experiment were measured in the same assay. The results were expressed either as a "total secretion" (the total amount of transferrin, secreted into both the apical (A) and the basal (B) compartments during the indicated period of time) or as a "basal/apical (B:A) ratio" (the ratio of the amount secreted into each compartment).

Testosterone was measured by radioimmunoassay in an aliquot of incubation medium, without previous extraction of steroids according to Caraty et al (1981). The usable range of the assay was 2–2.000 pg/tube with an intraassay coefficient of variation of 6%.

FSH was measured by a radioimmunoassay (Martinat and Combarnous, unpublished) in an aliquot of incubation medium. The rabbit anti-porcine FSH antibody was purchased from UCB (Liege, Belgique) and used at an initial dilution of 1/14.000. Reaction tubes contained 100 µl of either the standard or the incubation medium, which were diluted into phosphate buffer, pH 7.4, containing 0.1% Bovine Serum Albumin. Fifty microliters of iodinated (15.000 cpm) porcine FSH CY1208 (from Y Combarnous) and 100 µl of rabbit anti-porcine FSH serum containing 0.5% normal rabbit serum were added. After a 16 h incubation, at 20 C, 25 µl of sheep antiserum in rabbit γ-globulin and 1 ml of 6% polyethylene glycol in water were added in each tube and left for 15 min at room temperature. Then, tubes were centrifuged at 3.000 g for 20 min at 4 C. The radioactivity in the pellet was determined using a LKB Gamma counter. The usable range of the assay was 0.05–6 ng/tube, with an intraassay coefficient of variation of 8%. The samples were assayed in duplicate.

Colorimetric Assay of Inulin and Peroxidase

Inulin was measured by a microscale colorimetric assay. Inulin reacted with resorcinol in the presence of 37% chlorhydric acid solution. In a U-shaped well of a 96-well microtiter plate, 0.8–8 µl samples in 20 µl of water were added to 25 µl of resorcinol (5 mg/ml) and 150 µl of 37% chlorhydric acid. The solution was homogenized by shaking. The plates were heated at 85 C in an oven for 30 min. They were subsequently left in the dark at room temperature for 30 min. The optical density of each well was automatically recorded using a microtiter plate reader equipped with an interferential filter at 450 nm. Blank, neutral sugar standard and samples were assayed in duplicate. The usable range of the assay was 0.5–15 µg/20 µl.

Peroxidase was measured by a microscale colorimetric assay. The peroxidase reacted with 2,2'Azino-di-3 ethyl Benzo Thiazoline Sulfonate (ABTS) in the presence of 2% hydrogen peroxide (H_2O_2). In a U-shaped well of a 96-well microtiter plate, 2.5–25 µl samples in 50 µl of water were added to 100 µl containing 11 µg ABTS and 0.2 % H_2O_2 in citrate buffer 0.05 M, pH 4. The solution was homogenized by shaking. The plates were incubated in the dark at 37 C, for 45 min. Then, 20 µl of sodium Dodecyl Sulfate (SDS, 10%) were added. The optical density of each well was automatically recorded by using a microtiter plate reader equipped with an interferential filter at 405 nm. Blanks, standards, and samples were assayed in duplicate. The usable range of the assay was 0.1–20 ng/well.

Results

Morphology

Sertoli cells from 18-day-old rats attached to the Millipore filter within 2–3 h and formed a confluent monolayer within 20–24 h after plating. Contaminating peritubular cells and spermatogonia were inserted at random in the Sertoli cell monolayer. Nevertheless, the Sertoli cells grown on a permeable filter were more cuboidal and the nuclei more ovoid than those grown on plastic. Although cells grown at high density (1.5×10^6 cells/cm^2) on a filter without extracellular matrix assumed a cuboidal shape, they still did not resemble Sertoli cells *in vivo*. A number of cytoplasmic extensions containing mitochondria were observed inside the filter (Fig. 3A). However, when extracellular matrix was combined with a permeable filter support and a high plating density (1.5×10^6 cells/cm^2), Sertoli cells became tall and columnar with a characteristic nucleus, large lipid inclusions, and slender rod-shaped mitochondria in the apical cytoplasmic stalk that were oriented in a parallel direction to the long axis of the cell. In addition, Sertoli cells maintained contaminating spermatogonia in the basal part of the monolayer. Spermatocytes were excluded, and peritubular cells crept between Sertoli cells and the extracellular matrix (Fig. 3B). Sertoli cell tight junctional complexes were observed in monolayer after 5 days of culture (Fig. 3C).

Permeability Studies

After a 5-day stationary culture period in two experiments using a flow rate of 9 ml/h for a 6 h superfusion, the passage of inulin, peroxidase, and FSH across the filter without either cells or extracellular matrix was approximately 10–20% (Fig. 4). When Sertoli cells or Leydig cells were cultured direclty on the filter, the passage of inulin, peroxidase, and FSH was blocked, and that of testosterone was reduced to 3–4% for Sertoli cells and blocked for Leydig cells (Fig. 4). The extracellular matrix alone (210 g/cm^2) slightly and non-specifically decreased the passage of the different substances (Fig. 5). Sertoli cells grown on extracellular matrix blocked inulin, peroxidase, and FSH diffusion, but did not affect the passage of testosterone (Fig. 5). Leydig cells grown on extracellular matrix did not decrease the passage of inulin, peroxidase, FSH or

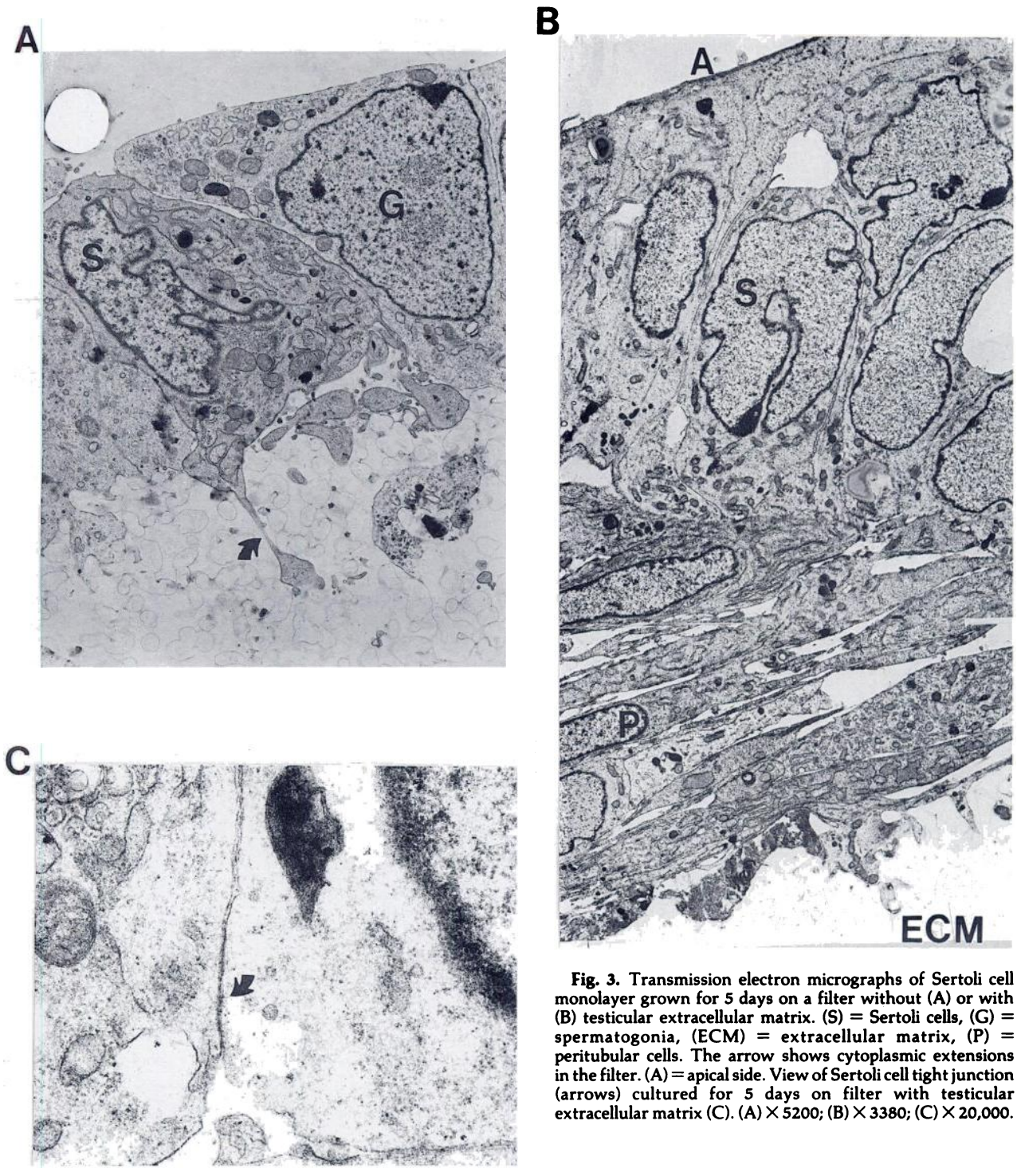

Fig. 3. Transmission electron micrographs of Sertoli cell monolayer grown for 5 days on a filter without (A) or with (B) testicular extracellular matrix. (S) = Sertoli cells, (G) = spermatogonia, (ECM) = extracellular matrix, (P) = peritubular cells. The arrow shows cytoplasmic extensions in the filter. (A) = apical side. View of Sertoli cell tight junction (arrows) cultured for 5 days on filter with testicular extracellular matrix (C). (A) × 5200; (B) × 3380; (C) × 20,000.

testosterone (Fig. 5), but delayed the diffusion into the apical compartment.

With a flow rate of 1.7 ml/h and a filter covered with extracellular matrix alone, 27 ± 4% (n = 3) of peroxidase (Fig. 6A) and 22 ± 2% (n = 3) of inulin (Fig. 6B) that perfused in the basal compartment were measured in the apical compartment. After a 2-day stationary culture period, Sertoli cells grown on extracellular matrix reduced the passage of peroxidase to 5 ± 2% (n = 4) (Fig. 6A) and the passage

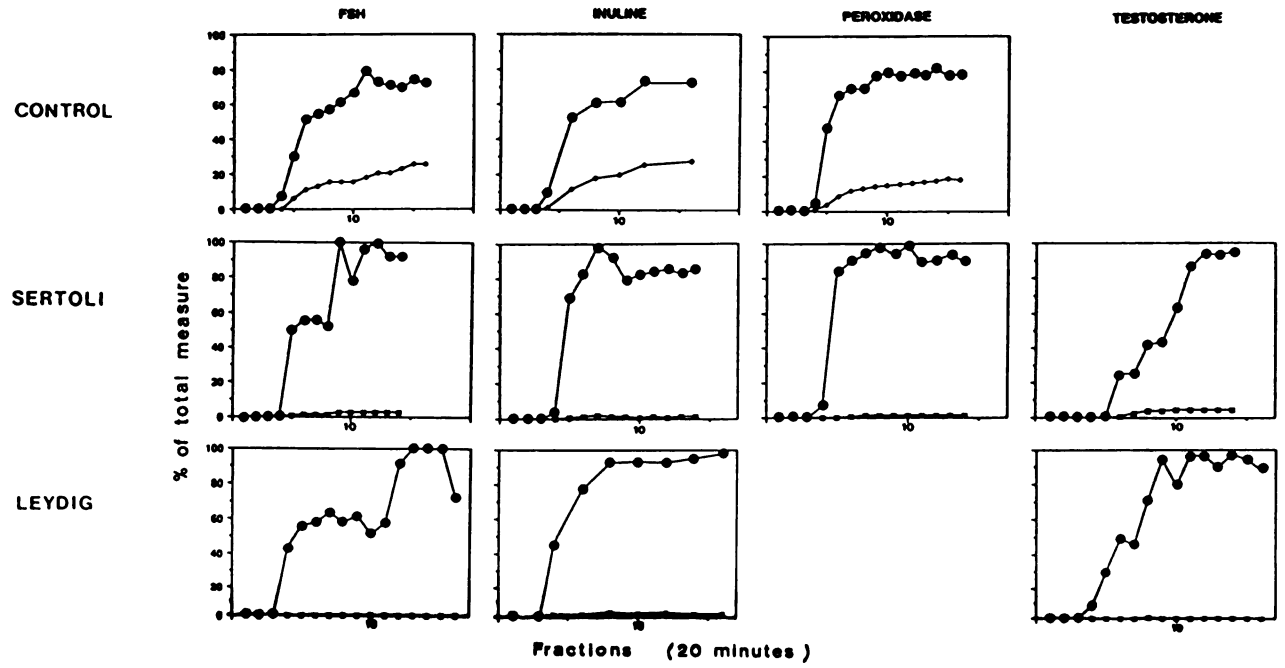

Fig. 4. Time course of FSH, inulin, peroxidase, and testosterone passing from the basal to the apical chamber. After a 5-day stationary culture, the system was superfused for 6 h at a flow rate of 9 ml/h with FSH, inulin, peroxidase, and testosterone in the medium of the basal compartment. Passage across the filter alone, plus Sertoli cells or plus Leydig cells, (●—●) basal compartment (◆—◆) apical compartment. Representative experiments among two superfusions assayed in duplicate. Y = axis represents the percentage of the total substance perfused into the basal compartment that was recovered in the basal and the apical compartments.

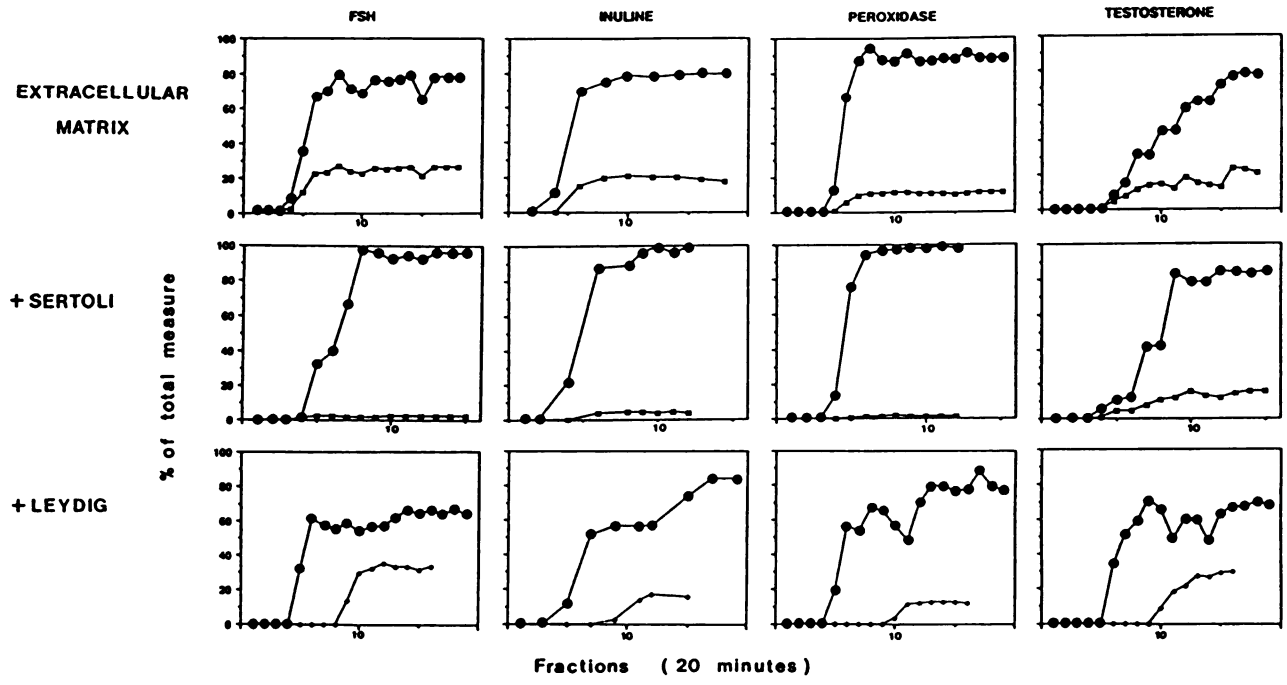

Fig. 5. Passage across the filter impregnated with the testicular extracellular matrix, plus Sertoli cells or plus Leydig cells. Symbols explained in legend for Figure 4.

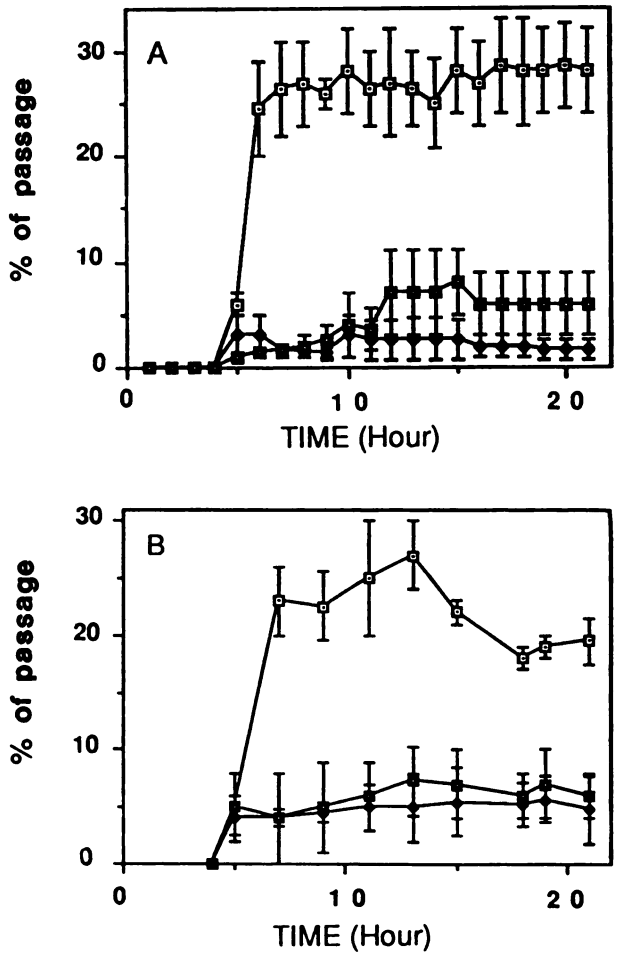

Fig. 6. Time course of peroxidase (A) and inulin (B) passing from the basal to the apical chamber superfused at a flow rate of 1.7 ml/h for 20 h across a filter covered with only testicular extracellular matrix (□—□) or a filter covered with testicular extracellular matrix plus Sertoli cells, after 2 days (◆—◆) or 5 days (■—■) of stationary culture. The peroxidase and inulin were perfused into the basal chamber at time 0. Mean ± SD from three to four separate experiments that were assayed in duplicate. Y = axis represents the percentage of the total substance perfused into the basal compartment that was recovered in the apical compartment.

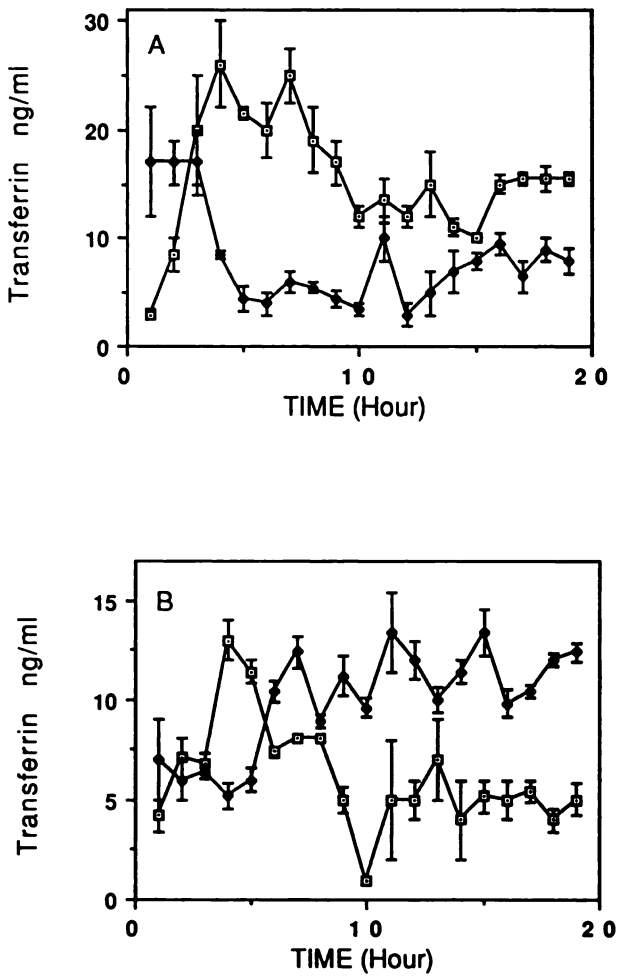

Fig. 7. Time course of transferrin secretion during 20 h superfusions after 2 days (A) or 5 days (B) of stationary culture. The basic medium 3F was perfused at a flow rate of 1.7 ml/h into the basal compartment at time 0. One representative experiment among three superfusions assayed in triplicate. (□—□) basal compartment, (◆—◆) apical compartment.

of inulin to 3 ± 2% (n = 4) (Fig. 6B). After a 5-day stationary culture period, the passage of these substances was of the same order of magnitude (5 ± 3% and 6 ± 3% [n = 3] respectively). Equilibrium of the two substances in the apical and basal compartments was reached at 4 hours in all experiments.

Effect of the Day of Culture on the Pattern of Transferrin Secretion

Figure 7A shows a representative experiment among three superfusions on the pattern of transferrin secretion by Sertoli cells grown on extracellular matrix for 2 days and superfused for 20 h with basic medium plus 3 F in the basal compartment at a 1.7 ml/h flow rate. During the first 3 h of superfusion, transferrin secretion in the basal compartment gradually increased from 3 to 20–25 ng/ml. It was constant until 8 h and decreased between 8 and 10 h. Afterwards, a relatively constant secretion (10–15 ng/ml) was observed. In the apical compartment, transferrin secretion was 15–20 ng/ml until 3 h and it decreased between 3 and 5 h. Afterwards, a relatively constant secretion (3–10 ng/ml) was observed.

Figure 7B shows a representative experiment among three superfusions on the pattern of

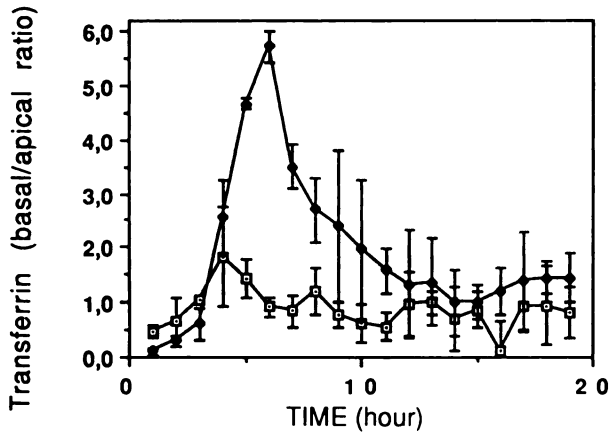

Fig. 8. Time course of the basal upon apical ratio of transferrin secretion by Sertoli cells, during a 20 h superfusion with basic medium 3 F after 2 days (◆—◆) or 5 days (□—□) of stationary culture. Mean ± SD from three separate experiments that were assayed in triplicate.

transferrin secretion by Sertoli cells grown on extracellular matrix for 5 days and superfused for 20 h with basic medium plus 3 F in the basal compartment at a 1.7 ml/h flow rate. During the first 4 h of superfusion, transferrin secretion in the basal compartment increased. It then decreased gradually for 6 h; a relative constant secretion was observed afterwards (4–6 ng/ml). In the apical compartment, transferrin secretion was relatively constant for 5 h. Then, it increased to 10–13 ng/ml and remained constant during the superfusion.

Total transferrin secretion during the 20 h of superfusion after 2 or 5 days of stationary culture was similar (542 ± 153 ng and 678 ± 176 ng, respectively; n = 3), but the distribution of transferrin secretion between the basal and apical compartments during the superfusion was different. After 2 days of stationary culture, the amount of transferrin secreted for 20 h of superfusion was 208 ± 19 ng in the apical compartment and 334 ± 30 ng in the basal compartment (n = 3). After 5 days of stationary culture, the amount of transferrin secreted for 20 h was 379 ± 85 ng in the apical compartment and 300 ± 82 ng in the basal compartment (n = 3). The average basal/apical ratio for total transferrin secreted during 20 h of superfusion was 1.6 ± 0.60 after 2 days of stationary culture and 0.79 ± 0.14 after 5 days.

Figure 8 shows the evolution of the basal/apical ratio of transferrin secretion during 20 h of superfusion at a flow rate 1.7 ml/h with the basic medium 3 F. After 2 days of stationary culture, the basal/apical ratio was 0.5 for the first 3 h, and it increased rapidly until 6 h (B/A = 5–6). It then decreased slowly between 6 and 12 h, and remained relatively constant afterwards (B/A = 1–2). In contrast, after 5 days of stationary culture, the basal/apical ratio increased a little between 3 and 5 hours (B/A = 1.5–2) and was then relatively constant afterwards (B/A = 0.5–0.8).

Discussion

The present paper describes a new two-compartment superfusion system which was specifically developed for the kinetic study of Sertoli cell secretion under various experimental conditions. In this system, the polarization and transport functions can be monitored continuously. Moreover, hormones can be delivered in a continuous or pulsatile manner to either the basal, the apical, or both sides of the cell monolayer. A similar system was reported by Janecki et al (1987) for a Sertoli cell monolayer cultured in a two-compartment chamber on a Millipore filter, but the patterns of transferrin and Androgen Binding Protein secretions were determined from 12 h samples collected for 12 days. In our system, the profile of transferrin secretion was determined from 1 h samples collected for 20 h. The cells formed a confluent monolayer and remained viable and functional under the superfusion culture conditions. All cells were uniformly exposed to the factors tested. The medium was delivered in apical and basal compartments at a similar rate. The cultures were maintained in a stable environment (temperature and pH).

The inter- and intra-experimental variations in medium flow rate were small. The hydrostatic pressure should be equal on both sides of the monolayer to prevent uncontrolled medium flow across the filter, but this was difficult to achieve in practice. The high filter area and low flow resistance of the filter alone caused instantaneous fluid shifts across the filter, even after small changes in either flow resistance or flow rate in one of the compartments. Although confluent Sertoli cell monolayers drastically reduced these phenomenon, incidental medium shifts still occurred. To solve this problem, I used an equivalent system called "the collecting unit," described by Janecki et al (1987). Under equilibrium condition, media pumped simultaneously through both compartments at similar rates would accumulate in the corresponding collecting units. The medium columns in the units should be of equal height. Any distubance of the

flow rate in either compartment results in different fluid levels in the collecting units. These differences quickly equalize the hydrostatic pressure in the two compartments. This system is very efficient in maintaining the effluent fluid pressures from the adjacent compartments.

Under our conditions of culture, one might expect the cell morphology and behavior to be different from those in monolayer cultures grown on plastic surfaces. Sertoli cells growing at high density on a filter that is not impregnated with extracellular matrix have a more cuboidal morphology, and the nuclei are centrally located, irregularly oval, or pyramidal. Contaminating peritubular cells and spermatogonia are inserted at random between the Sertoli cells. Sertoli cells, however, send processes deeply into the filter. This phenomenon was also observed for Leydig cells, BMK cell line (results not shown), and other epithelial cells (Hay, 1981). On the other hand, Sertoli cells grown at same density on a filter impregnated with testicular extracellular matrix do not present any disorganized basal cytoplasm nor cell processes into the pores of the filter. They form tall columnar monolayers with ovoid or pyramidal nuclei and tight junctions predominantly located in the basal part of the cytoplasm. In addition, they maintain contaminating spermatogonia in the basal part of the monolayer. Similar morphological features were reported by Byers et al (1986). Although the number of peritubular cells was similar after 2 or 5 days of stationary culture with or without extracellular matrix, I observed that peritubular cells present in Sertoli cell preparations were always located between the extracellular matrix and Sertoli cells. Peritubular cells seem to have a high affinity for extracellular matrix purified from mature rat testes. The association of the different cellular types in this culture system form an architecture somewhat analogous to the seminiferous epithelium.

If polarized secretion or transport through the monolayer has to be tested, Sertoli cells should form a tight confluent monolayer. We have observed that Sertoli and Leydig cells grown on a filter without testicular extracellular matrix totally restricted the transepithelial passage of macromolecule and testosterone. Similar results were reported for inulin by Janecki and Steinberger (1986) using Sertoli cell culture in a stationary two-compartment chamber. The cell processes observed by transmission electron microscopy inside the filter pores increase with culture time and finally clog up the pores of the filter and create an artificial barrier. On the other hand, Sertoli cells grown on a filter impregnated with testicular extracellular matrix form a monolayer which is specifically impermeable to inulin, peroxidase, and FSH, but not to testosterone. Similar results for inulin were reported by Byers et al (1986) and Ailenberg et al (1988), using Sertoli cells in a stationary two-compartment culture chamber. Leydig cells grown on the filter impregnated with extracellular matrix did not restrict the diffusion of inulin, peroxidase, FSH, and testosterone into the apical chamber. Thus, Sertoli cells form a selective barrier somewhat analogous to the blood testis barrier (Setchell and Brooks, 1988). The decreasing permeability of the Sertoli cell monolayer on extracellular matrix might be due to the formation of tight junctions. In the rat, Sertoli cell tight junctions appear between 16 and 18 days of postnatal life (Vitale et al, 1973). Thus, the appearance of tight junctions in cultures of cells from 18-day-old animals could represent regeneration of junctions that were disrupted during the cell isolation procedure. Changing the medium flow rate from 1.7 to 9 ml/hour did not significantly influence the regeneration of junctions. Complete junction formation in monolayers is FSH and testosterone independent and was obtained rapidly within 1–2 days of culture. The permeability of the epithelial sheet cannot be changed by testicular myoid cells, since myoid cells do not form occluding junctions (Dym and Fawcett, 1970) and do not clog up the pores of the filter by cell processes. Finally, electron microscopy confirmed the presence of tight junctions between cultured Sertoli cells derived from 18-day-old animals, as observed by Byers et al (1986) and Janecki et al (1987).

I also investigated the influence of the preliminary culture duration on the bidirectional secretion of transferrin. After 2 days of stationary culture, approximately twice as much transferrin was secreted into the basal compartment as into the apical compartment after a 20 h superfusion. Mean B/A ratio was 1.6 ± 0.6. This ratio was reduced to 0.8 ± 0.14 after 5 days of stationary culture. Such a difference was due to an increase in transferrin secretion in the apical compartment after 5 days of culture. In contrast, the total amounts of transferrin after a 20 h superfusion in the basal compartment was the same after 2 or 5 days of stationary culture. These differences are not representative of the basal/apical ratio variations during the superfusion. After 2 days of stationary culture, the basal/apical ratio

increased rapidly during the first 6 h of superfusion with basic medium plus 3 F and then decreased slowly. On the other hand, after 5 days of stationary culture, the basal/apical ratio was relatively constant during the superfusion period. The origin of this phenomenon is unknown. It is difficult to compare our observations with what is known about the polarized secretion of transferrin by Sertoli cells *in vitro*. Differences between stationary and superfused culture chambers and the complexity in the kinetics of diffusion in the two chamber system may contribute to discrepancies between different investigators concerning relative levels of transferrin present in the apical and basal compartments. In the stationary two-chamber culture systems used by Hadley et al (1987), where cells were plated on gelated Matrigel, higher concentrations and total levels of transferrin were present in the apical chamber, corresponding to an apparent adluminal preferential secretion. In contrast, according to Janecki and Steinberger (1987a) and Ailenberg et al (1988), higher total levels of transferrin were secreted into the basal chamber, corresponding to apparent basal preferential secretion. This was especially pronounced in the presence of peritubular cells or serum (Janecki and Steinberger, 1987b; Ailenberg et al, 1988). The media employed were of somewhat different composition, the collection periods and treatments were different, and the volumes of the two chamber assemblies varied. In addition, one group employed hydrated (gelated) forms of Matrigel, whereas the other group employed a filter, uncoated and coated with air-dried Matrigel (Janecki and Steinberger, 1986; Hadley et al, 1987; Ueda et al, 1988; Ailenberg et al, 1988). Two groups used the same volume for the two compartments, whereas the other group used a basal compartment 12 times larger than the apical compartment (Ailenberg et al, 1988 and 1989). In a superfused culture chamber, it is difficult to compare our observations with Janecki et al (1987), due to differences in collection periods, time of culture, and the fact that the filter was not coated with extracellular matrix. These authors observed that the addition of serum, FSH, and testosterone enhanced total levels of transferrin secreted into the basal chamber, which agrees with our results.

From the literature, it is obvious that rat ABP is secreted bidirectionally *in vivo* and that polarity of this secretion changes during testicular maturation (Mather et al, 1983). In adult animals, approximately 80% of total secreted ABP is directed toward the lumen and only 20% is secreted into the interstitial fluid. However, before junction formation or after treatments causing damage of the adult seminiferous epithelium, a greater proportion of ABP is secreted into the interstitial fluid (Gunsalus et al, 1980). Nothing is known about the *in vivo* secretion of transferrin. There is only indirect evidence that it is also bidirectional (Holmes et al, 1983). *In vitro* transferrin secretion is bidirectional but may be regulated differently from ABP (Perez-Infante et al, 1986). In addition, the presence of extracellular matrix was found to cause a 25% increase in the basal level of transferrin production, but it had no effect on the basal level of ABP production (Anthony and Skinner, 1989). The meiotic germ cells that appear at the time of blood testis barrier formation may change the direction of ABP secretion to predominantly apical. The coculture of Sertoli cells with germ cells (> 85% pachytene spermatocytes) reversed the basal/apical ratio of ABP from 2 to 0.7 (Janecki et al, 1988). On the other hand, the addition of pachytene spermatocytes or conditioned medium did not change the basal/apical ratio of transferrin, in spite of an increase in the synthesis and secretion of total proteins (Janecki et al, 1988; Djakiew and Dym, 1988). Recently, Handelsman et al (1989) showed that in a stationary two chamber culture system, Sertoli cells cultured on Matrigel secreted 90% of inhibin into the apical compartment, and they suggested that inhibin secretion by immature rat Sertoli cells *in vivo* may be directed into the seminiferous tubular lumen and may also be important for spermatogenesis.

But *in vitro* polarity studies in two-compartment culture systems can be compared with *in vivo* studies only if Sertoli cell morphological and functional integrity is preserved. I observed, however, that Sertoli cells cultured on extracellular matrix (testicular or Matrigel) expressed a fetal cytokeratin intermediary filament. This fetal expression increases during culture time (Guillou, unpublished data). Cytosqueleton plays an important role in the transport of secretion products (Geiger, 1983 and Zor, 1983). The regulation of Sertoli cell polarized secretions is a complex process in which hormones, serum factors, and other cell types in the testis and Sertoli cell cytosqueleton may play a role. But experiments with Sertoli cells cultured in two-compartment systems should provide more precise information in this regard. Our superfusion two-compartment culture system not only offers more

physiological conditions for the cultured cells, but is an excellent model for investigating the influence of various hormonal stimuli on the secretory activity of Sertoli cells and on the efficiency of the blood testis barrier.

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