Pachytene spermatocyte and round spermatid binding to Sertoli cells 

in vitro

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Summary

Spermatogenic cells differentiate in vivo while in continuous contact with the Sertoli cell. During differentiation, the spermatogenic cells and Sertoli cells form a number of morphologically distinct stage-specific adhesions. We describe an in vitro assay system for studying the adhesion of spermatogenic cells to Sertoli cell monolayers. Mixed populations of spermatogenic cells or enriched fractions of pachytene spermatocytes and round spermatids were labelled with the vital dye, fluorescein diacetate, prior to their addition to Sertoli cell monolayers so that the adhesion of viable spermatogenic cells could be quantified. Using this assay system, the number of pachytene spermatocyte and round spermatid binding sites on the Sertoli cell monolayer were similar, but the kinetics of binding were different. Pachytene spermatocytes were able to inhibit significantly round spermatid binding, while round spermatids did not significantly inhibit pachytene spermatocyte binding. After coculture for 24–48 h, spermatocytes form junctional structures with Sertoli cells that are similar to desmosome-like junctions. These results suggest that pachytene spermatocytes and round spermatids bind to Sertoli cells by different mechanisms.

Key words: Sertoli cell, spermatogenic cell, adhesion.

Introduction

Spermatogenesis is the process of male germ cell proliferation and differentiation that occurs in continuous contact with a somatic cell, the Sertoli cell. Throughout differentiation the spermatogenic cells and the Sertoli cells share morphologically distinct plasma membrane junctions (for review, see Russell, 1980). There are at least five morphologically distinct cell–cell interactions, each present throughout certain stages of spermatogenic cell differentiation. Desmosome-like junctions are seen between the Sertoli cells and the following spermatogenic cells: spermatogonia, primary and secondary spermatocytes, and early round spermatids (Altorfer et al. 1974; Eddy & Kabrì, 1976; Kaya & Harrison, 1976; Russell, 1977a; Palombi et al. 1979; Romrell & Ross, 1979; Russell et al. 1983). Small gap junctions have been reported between Sertoli cells and premeiotic spermatogenic cells (McGinley et al. 1979; Szollosi & Marcaillou, 1980), but not between Sertoli cells and elongating spermatids. Ectoplasmic junctional specializations, which are unique to the testis, are formed between the Sertoli cell and round and elongating spermatids (Flickinger & Favcett, 1967; Ross & Dobler, 1973; Ross, 1976; Russell, 1977b, 1979; Romrell & Ross, 1979). Originally referred to as junctional specializations by Flickinger & Favcett (1967), and later as ectoplasmic specializations by Russell (1977b), they appear similar to half of a Sertoli–Sertoli cell junction within the Sertoli cell and form on the plasma membrane overlying the acrosomal membrane of the developing spermatid. These specializations hold elongating spermatids to Sertoli cells prior to release into the lumen (Russell, 1977b; Romrell & Ross, 1979) and thus will be referred to as ectoplasmic junctional specializations. Tubulobulbar complexes, which are long cytoplasmic extensions of spermatid cytoplasm held in deep recesses of the Sertoli cell, form only between elongating spermatids and Sertoli cells (Russell & Clermont, 1976; Russell, 1979; Russell & Malone, 1980). At spermiation, the release of the mature spermatids, a portion of the
residual cytoplasm, the residual body, is phagocytosed by the Sertoli cell (Fawcett & Phillips, 1969; Ross, 1976; Russell, 1984). The stage-specific nature of these plasma membrane specializations, and the stage-specific movement of spermatogenic cells in relationship to the apical/basal axis of the Sertoli cell, have led workers to propose that spermatogenic cells display stage-specific antigens on their surfaces that Sertoli cells recognize and to which they adhere (Millette & Bellvé, 1977; Ziparo et al., 1980; Millette & Scott, 1984).

In order to examine the complex cell-cell interactions occurring in vivo, simplified in vitro model systems have been developed. Using these systems, a number of workers have begun to examine the nature of the adhesion between Sertoli cells and spermatogenic cells (Ziparo et al., 1980; Palombi et al., 1980; Galdieri et al., 1981; Grootegoed et al., 1982; D'Agostino et al., 1984; Le Magueresse et al., 1986; Van Der Donk et al., 1986; D'Agostino & Stefani, 1987).

To our knowledge, the earliest report of de novo, in vitro adhesion of spermatogenic cells to Sertoli cells was that of Ziparo et al. (1980). Using the rat as a model system these workers reported that pachytene spermatocytes and round spermatids adhered to monolayers of Sertoli cells. However, the number of round spermatids that bound to the Sertoli cell monolayers was only about 20% of the number of pachytene spermatocytes that bound using their assay system. More recently, other authors have reported that they obtained a greater percentage of pachytene spermatocyte than round spermatid binding to enriched rat Sertoli cell monolayers (DePhilip, 1985; Le Magueresse et al., 1986). In this paper we report a new in vitro assay procedure for examining the binding of spermatogenic cells to enriched Sertoli cell monolayers using fluorescein diacetate to label viable, adherent germ cells. A preliminary report of some of these observations has been made in abstract form (Enders & Millette, 1984).

Materials and methods

Isolation and culture of primary Sertoli cells

Primary Sertoli cells were isolated from 16- to 18-day-old CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) according to a slight modification of the procedures of Mather & Phillips (1984) as described (Enders et al., 1986). Briefly, 1mM-glycine, 2mM-EDTA, 20-40ku. ml⁻¹ DNase, 0-2mg ml⁻¹ soybean trypsin inhibitor in Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS) was used to dissociate the interstitial cells from the seminiferous tubules. Peritubular myoid cells were then stripped from the tubules with 0.5mg ml⁻¹ collagenase/dispase (Boehhringer Mannheim Biochemicals, Indianapolis, IN), 0-05mgl⁻¹ soybean trypsin inhibitor, 0-01mg ml⁻¹ DNase in sterile Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 with l-glutamine, 15mM-Hepes buffer (Sigma Chemical Co., St Louis, MO), 10mg l⁻¹ gentamicin (Irving Scientific, Santa Ana, CA) (abbreviated DMEM/F-12). The collagenase/dispase mixture was also used to disrupt the isolated tubules into individual cells and small Sertoli cell aggregates. The enriched Sertoli cell fraction was washed twice in fresh DMEM/F-12 and plated in DMEM/F-12 plus 10% foetal calf serum (FCS, Gibco, Grand Island, NY), plus 3µg ml⁻¹ cytosine arabinoside (Sigma Chem. Co.), according to Tung et al. (1980), on glass coverslips. Early experiments determining the effect of temperature, time, and specificity of spermatogenic cell–Sertoli cell adhesion, did not include cytosine arabinoside in the media. Typically four, 18mm x 18mm coverslips were placed in 60mm x 15mm culture dishes, cells added at a concentration of 4x10⁵ to 8x10⁵ cells cm⁻² and incubated at 33°C in 95% air/5% CO₂ for 2 or 3 days prior to hypotonic lysis of the contaminating germ cells with 20nm-Tris-HCl, HCl, according to Galdieri et al. (1981). Generally, Sertoli cell monolayers were cultured for a total of 6–10 days before their use in the spermatogenic cell–Sertoli cell adhesion assay.

Isolation and culture of spermatogenic cells

Spermatogenic cell isolation and culturing was modelled after the procedures of Gerton & Millette (1984). Mixed populations of spermatogenic cells were isolated from 60- to 120-day-old TAC:(SW) fBR mice (Taconic Farms, Inc., Germantown, NY) according to the procedures of Romrell et al. (1976). In experiments examining the effects of temperature and time of incubation mixed populations of adult spermatogenic cells, containing all stages of spermatogenesis, were used: i.e. spermatagonia, primary and secondary spermatocytes, round spermatids, and residual bodies. In further experimentation enriched fractions of pachytene spermatocytes and round spermatids isolated by unit gravity sedimentation through a bovine serum albumin (BSA) gradient were used (Bellvé et al., 1977). Because large numbers of spermatogenic cells were needed for saturation studies, the purity of the pachytene spermatocyte and round spermatid fractions isolated by unit gravity sedimentation was accepted at 84–93%, slightly below that obtainable using these procedures if only the peak of each cell fraction were used. Both the mixed populations of adult spermatogenic cells and the purified pachytene spermatocyte and round spermatid fractions were washed in sterile DMEM/F-12 plus 5% (or occasionally 10%) FCS, 1mM-sodium pyruvate, 6mM-sodium lactate, 2mM-glutamine (referred to as PLG). Spermatogenic cells were cultured overnight at 33°C in 95% air/5% CO₂ in order to allow the cells to resynthesize any surface components that might have been cleared during their enzymic isolation (Millette & Moulding, 1981; Gerton & Millette, 1986).

In vitro spermatogenic cell–Sertoli cell adhesion assay

After overnight culture, spermatogenic cells were labelled with fluorescein diacetate (Mishell & Shiigi, 1980), a method that labels only cells with esterase activity and intact plasma membranes, so that only viable cells were counted. Then the labelled cells were washed twice in DMEM/F-12 plus PLG...
before being added to the Sertoli cell monolayers. During the washing procedures the spermatogenic cell numbers were adjusted so that 1 ml of medium was added to 9 ml of DMEM/F-12 plus 10% FCS already present in the 60 mm × 15 mm culture dishes containing the coverslips. The culture dishes were then placed in a tray on an orbital shaker table (≈30 cycles min⁻¹, = 1 inch of rotation) for 60 min at room temperature. Alternative procedures are described in Results. At 15-min intervals the tray containing the culture dishes was gently agitated linearly by hand to redistribute cells from the centre of the dishes. The tray was used to ensure that each dish received the same agitation. After 60 min the coverslips were removed from the culture dishes and placed into DMEM/F-12 in coverslip Coplin jars. To remove loosely adherent spermatogenic cells, each coverslip was dropped from just above the surface of the DMEM/F-12 twice into three separate Coplin jars. The coverslips were then carefully placed cell side down on slides in a humidified box kept at 4°C or on ice until examined (generally within 4 h) for epifluorescence with fluorescein filter combinations. The total number of fluorescently labelled cells in 10 randomly chosen fields (6·3× or 10× objective) on each coverslip was counted, resulting in four instances, especially when large numbers of adherent cells were present, each field was photographed or the video image was stored on laser disk for later analysis. In these cases the number of cells in 8–10 fields on four coverslips was independently recorded. The statistical significance of the results was determined by using Student’s t-test.

Ultrastructural techniques
In order to examine the nature of the adhesion between spermatogenic cells and Sertoli cells at the ultrastructural level, mixed populations of spermatogenic cells were added to Sertoli cell monolayers, which were grown directly on tissue culture plastic as opposed to coverslips. Mixed populations of spermatogenic cells were added to the Sertoli cell monolayers and gently shaken as described above. Non-adherent spermatogenic cells were removed by rinsing the culture dishes three to four times with fresh DMEM/F-12, and then placed in DMEM/F-12 plus 10% FCS and cultured for 24–48 h. The medium was then removed and the cells fixed with 1% paraformaldehyde, 3% glutaraldehyde in 0·1 M-sodium cacodylate buffer, pH 7·4–5. The cells were postfixed in 1% OsO₄, en bloc stained in uranyl acetate, and dehydrated in ethanol. A stream of propylene oxide was used to dissolve a portion of the tissue culture dish to permit removal of the cells. Dissolved polystyrene was removed by washing the cells four to six times in propylene oxide prior to infiltration and embedding in an Epon/Araldite mixture. Thin sections were cut and stained with uranyl acetate and lead citrate prior to examining.

Results

Enriched Sertoli cell monolayers
The Sertoli cell plating density (4×10⁵ to 8×10⁵ cm⁻²) resulted in Sertoli cell monolayers that completely covered the glass coverslip when examined by phase-contrast microscopy. Acridine Orange was occasionally used to aid the process of cell counting and to examine for myoid cell contamination. Sertoli cells are easily distinguishable from peritubular myoid cells because of their extensive perinuclear acidic compartments. Local cell density varied, but generally was between 1·2×10⁶ and 5×10⁶ cells cm⁻², meaning individual Sertoli cells occupied between 1600 and 8300μm², and thus are much more squamous than Sertoli cells in vivo.

Effect of temperature
Mixed populations of cultured spermatogenic cells (not separated by unit gravity sedimentation, but cultured overnight) were used to examine the effect that temperature had on adhesion of spermatogenic cells to enriched Sertoli cell monolayers. It was found that similar numbers of mixed germ cells bound to the Sertoli cell monolayer when incubated at either 24°C or 33°C, while incubation at 4°C significantly reduced binding (Fig. 1). If the culture dishes were gently shaken at room temperature during the incubation period of 60 min, a significant increase in the number of adherent spermatogenic cells was observed. Thus, all future incubations were performed with gentle agitation at room temperature.

Effect of time on adhesion
The effect of incubation time on spermatogenic cell–Sertoli cell adhesion was examined. When mixed populations of spermatogenic cells were gently agitated on top of enriched Sertoli cell monolayers at room temperature, cell binding peaked after about one hour of incubation (Fig. 2). Two additional experiments (not illustrated), which continued the incubation periods for up to 5 h, did not show an increase in spermatogenic cell binding above that observed at 60 or 120 min at either 24 or 33°C.

Fig. 1. Both temperature and gentle shaking affect the adhesion of mixed spermatogenic cells to Sertoli cell monolayers. 0·93×10⁶ mixed spermatogenic cells per cm² were added to Sertoli cells. The binding at 4°C and at 24°C when shaken is significantly different from binding at either 24 or 33°C, * = P < 0·012. In this experiment n = 3. The error bars = standard error of the mean.
Effect of time of incubation

Time of incubation (min)

No. of cells bound/field

Specificity of cell binding

Different substrata

No. of cells bound/field

Fig. 2. The time of incubations of spermatogenic cells with the enriched Sertoli cell monolayers affects the number of spermatogenic cells that bind to the Sertoli cell monolayer. 3.6 \times 10^5 spermatogenic cells per cm$^2$ were added to Sertoli cell monolayer. In this experiment $n = 6$. The error bars = standard error of the mean.

90 min. Thus, for all the following experiments the adhesion assay was performed at room temperature with gentle agitation, for 60 min.

Specificity of the adhesion

The specificity of spermatogenic cell–Sertoli cell adhesion was examined by placing either pachytene spermatocytes or round spermatids on other substrata. Both spermatogenic cell types showed little affinity for either 3T3 cells, bare coverslips, or the enriched Sertoli cell monolayer if the spermatogenic cells were first trypsinized (Fig. 3). In control preparations the pachytene spermatocytes and round spermatids cells that adhered to the Sertoli cell monolayer generally bound with a seemingly random distribution to the Sertoli cells (Figs 4, 5). While some clumping of cells was seen, the cell clumps were counted as single cells.

Saturation binding studies

The numbers of pachytene spermatocytes and round spermatids added to enriched Sertoli cell monolayers were varied over a wide range to examine the binding kinetics of the two cell types (Fig. 6). When the total number of spermatogenic cells added was low (below \( \approx 5 \times 10^5 \text{ cm}^{-2} \)), a greater percentage of pachytene spermatocytes bound than round spermatids in eight out of 10 trials in eight different experiments (different Sertoli cells and spermatogenic cells on different dates). The data are not illustrated. However, when the number of spermatogenic cells added was large (above \( \approx 5 \times 10^5 \text{ cm}^{-2} \)), the total number of round spermatids that bound often exceeded the number of pachytene spermatocytes that bound (Fig. 7; Table 1).

In four out of six separate experiments when more than \( 5 \times 10^5 \text{ cells cm}^{-2} \) were added to enriched Sertoli cell monolayers, the number of round spermatids that bound was equal to or greater than the number of pachytene spermatocytes binding (data not illustrated).

Competitive binding studies

In order to determine if binding of pachytene spermatocytes and round spermatids occurred independently of the presence of each other, the following experiments were performed. A given number of pachytene spermatocytes that had been labelled with fluorescein diacetate were added to the enriched Sertoli cell monolayers along with twice the number of unlabelled round spermatids. The reciprocal experiment was also performed; that is, fluorescently labelled round spermatids were added to the Sertoli cell monolayer along with twice the number of unlabelled pachytene spermatocytes. Unlabelled pachytene spermatocytes were able to inhibit in a statistically significant manner labelled round spermatid binding (Fig. 7). However, the converse was not true. Unlabelled round spermatids did not significantly inhibit the binding of labelled pachytene spermatocytes. In four separate experiments pachytene spermatocyte binding was reduced an average of 11% by unlabelled round spermatids, while round spermatid binding was reduced an average of 72% by the addition of unlabelled pachytene spermatocytes (Table 1).
Fig. 4. Fluorescent photomicrograph of fluorescein diacetate-labelled pachytene spermatocytes adhering to an enriched Sertoli cell monolayer. Only cells that fluoresced strongly were counted. The percentage of fluorescent pachytene spermatocytes was generally 88–94%. Clumps of cells were counted as one cell, not two or several. ×49.

Fig. 5. Fluorescent photomicrograph of fluorescein diacetate-labelled round spermatids adhering to an enriched Sertoli cell monolayer. The intensity of the round spermatids was more variable than that of the pachytene spermatocytes. Typically 84–91% of the round spermatids fluoresced. Faintly fluorescent cells were not counted and clumps of round spermatids (there are at least 16 shown here) were counted as one adherent cell. ×49.

Effect of cell number

![Graph showing the effect of cell number on the number of cells bound/field.](image)

Fig. 6. The total number of pachytene spermatocytes (○) and round spermatids (●) which bound to Sertoli cell monolayers saturated at similar levels. At low cell numbers (below ≈5×10⁵ cells added per cm²) a greater percentage of pachytene spermatocytes bound than round spermatids. In other experiments not illustrated the saturating level of round spermatids to bind exceeded that of pachytene spermatocytes. In this experiment n = 4. The error bars = standard error of the mean.

Ultrastructural observations

Despite the fact that mixed populations of spermatogenic cells were added to the Sertoli cell monolayers, by the time the cells were cocultured for 24–48 h, fixed, and processed for thin-section electron microscopy, spermatocytes were the exclusive cell type observed adhering to the Sertoli cell monolayer (Fig. 8). Spermatocytes were typically held to underlying Sertoli cells by regions of cell–cell contact that displayed small quantities of electron-dense extracellular material between adjacent cells. In some instances the areas of Sertoli cell–spermatocyte contact also displayed focal increases in the electron density of Sertoli cell cytoplasm (Fig. 9). Endoplasmic reticulum was not associated with the regions of cell–cell contact. Spermatids were not observed adhering to the Sertoli cell monolayers.

Discussion

The cell–cell adhesion assay we introduce in this paper has a number of unique features not present in other
assays used to examine Sertoli cell–spermatogen cell interactions. Our assay is a fast, quantitative method (time of incubation is only 1 h) that does not rely on the use of radioisotopes. Most other Sertoli cell–spermatogenic cell adhesion assays used require incubation periods of 3–24 h or more (Ziparo et al. 1980; Tres & Kierszenbaum, 1983; Galdieri et al. 1984; Le Magueresse et al. 1986), with the exception of the assay of Grootegoed et al. (1982), who used concanavalin A to induce adhesion, and that of D’Agostino et al. (1984), who reported that pachytene spermatocyte binding to rat Sertoli cells saturated after 90 min at 33°C. The spermatogenic cell–Sertoli cell adhesion in our assay may require less time than others have reported for a number of reasons. First, we use gentle agitation during our assay to increase the number of cell–cell collisions during the assay. Second, we preincubate our spermatogenic cells overnight in order to allow them to resynthesize surface components that may have been cleaved enzymically during the isolation procedure. This is important, since the enzymatic treatment used to isolate the various cell types destroys the cell–cell adhesion present in vivo. Third, our assay uses mouse cells, while most previous work has been done using rat cells, which may display species differences. Moreover, our assay procedure is designed to examine the early recognition events between Sertoli cells and spermatogenic cells, while much of the previously described work was designed to study other aspects of Sertoli cell–spermatogenic cell interactions, such as changes in Sertoli cell secretory activity in response to spermatogenic cells (Le Magueresse et al. 1986).

During the development of this assay we had trouble with variability, both within a given experiment, and from one experiment to the next. Much of the intralexperiment variability was reduced by paying close attention to the details of the assay. However, the absolute number of spermatogenic cells adhering to a given Sertoli cell monolayer preparation still tends to vary from one experiment to the next, despite the fact that the relative trends in binding (e.g. pachytene spermatocytes versus round spermatids) was highly reproducible. Within the literature descriptions of spermatogenic cell–Sertoli cell adhesion are very variable. Ziparo et al. (1980) reported that about 90% of pachytene spermatocytes but only 10–30% of round spermatids bound to Sertoli cell monolayers when cell numbers added were below 4x10^5 cells cm^-2. Rivarola et al. (1985) reported that only 20–40% of the germ cells added (5x10^5 cell ml^-1) adhered to Sertoli cell monolayers after coculture periods of 4 and 24 h. They state that in their assay system “very few germ cells were observed on the surface of Sertoli cells after collection” (of the spermatogenic cells). Grootegoed et al. (1982), using mouse pachytene spermatocytes and round spermatids, reported that only 10–15% of cells initially added (2.5x10^5 cells cm^-2) to the Sertoli cells bound to the monolayers after coculture for up to 20 h in medium 199.

Previous investigations using both pachytene spermatocyte and round spermatid cells in binding studies reported that a greater percentage of added pachytene spermatocytes versus round spermatids were bound (Ziparo et al. 1980; Galdieri et al. 1984; Le Magueresse, 1986; D’Agostino & Stefanini, 1987). In these experiments spermatogenic cell densities of 4x10^5 cm^-2 or less were generally used. We found similar results at these cell densities. However, at higher germ cell density (5x10^5 cm^-2 or more), equal or greater numbers of round spermatids than pachytene spermatocytes were bound. The slope of round spermatids binding versus the number of cells added was less than that for pachytene spermatocytes, suggesting that Sertoli cells have a lower affinity for round spermatids than pachytene spermatocytes, but similar or slightly greater number of binding sites for round spermatids. In vivo each pachytene spermatocyte gives rise to four round spermatids, suggesting that on

### Table 1. Competitive binding assay

<table>
<thead>
<tr>
<th>Experiment (no. of labelled germ cells added/cm²)</th>
<th>Number of germ cells bound/field to Sertoli cell monolayers</th>
<th>% PSC binding reduced by +2xUnRST</th>
<th>% RST binding reduced by +2xUnPSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (6.25x10^6)</td>
<td>PSC 67 ± 5, PSC+2xUnRST 53 ± 9, RST 111 ± 10, RST+2xUnPSC 27 ± 3</td>
<td>21 %</td>
<td>76 %**</td>
</tr>
<tr>
<td>2 (8.375x10^6)</td>
<td>PSC 75 ± 4, PSC+2xUnRST 67 ± 5, RST 86 ± 5, RST+2xUnPSC 40 ± 4</td>
<td>34 %</td>
<td>54 %**</td>
</tr>
<tr>
<td>3 (9.875x10^6)</td>
<td>PSC 51 ± 3, PSC+2xUnRST 53 ± 3, RST 85 ± 9, RST+2xUnPSC 26 ± 2</td>
<td>34 %</td>
<td>70 %**</td>
</tr>
<tr>
<td>4 (9.625x10^6)</td>
<td>PSC 244 ± 56, PSC+2xUnRST 233 ± 33, RST 250 ± 56, RST+2xUnPSC 35 ± 12</td>
<td>4 %</td>
<td>86 %**</td>
</tr>
</tbody>
</table>

PSC, pachytene spermatocyte; RST, round spermatids; Un, unlabelled spermatogenic cells. Values are mean ± standard error of the mean; ( ), increased binding; n, the number of microscopic fields counted (experiments 1–3) or the sum of 10 microscopic fields (experiment 4). * = P < 0.006; ** = P < 0.0001; comparing RST binding with RST+UnPSC binding.
average each Sertoli cell should bind four times as many round spermatids as pachytene spermatocytes. Indeed, morphometric study of the rat testis indicates that there are 2-4 pachytene or diplotene spermatocytes and 7-9 step 1–10 round spermatids per rat Sertoli cell (Wing & Christensen, 1982). Yet a given Sertoli cell

**Fig. 8.** A pachytene spermatocyte (p) adhering to a Sertoli cell (S) grown on tissue culture plastic. The pachytene spermatocyte was cocultured with the Sertoli cell for 24 h prior to fixation. Note, while there are several regions of contact, the only specialization appears to be a slight increase in the electron density of material between the two adjacent phospholipid bilayers, and this only occurs in focal spots. ×12,000.

**Fig. 9.** A spermatocyte adhering to a Sertoli cell (S) grown on tissue culture plastic cocultured for 24 h. Note the increase in electron density of the extracellular material between the two plasma membranes of adjacent cells. There is also an increase in the electron density of the cytoplasm of the Sertoli cell adjacent to the regions of cell–cell contact (arrowheads). ×25,000.
makes many more cell–cell contacts, since diploid spermatogenic cells are frequently in contact with more than one Sertoli cell. The stage V Sertoli cell that Russell and coworkers (Russell et al. 1983) semiserial sectioned made contact with eight pachytene spermatocytes and 27 step 5 round spermatids. Therefore, it is somewhat surprising that previous workers have reported larger numbers of pachytene spermatocyte binding sites than those for round spermatids. This result may be due to a limited ability of round spermatids to resynthesize surface components responsible for cell binding compared to that of pachytene spermatocytes. Gerton & Millette (1986) have, however, demonstrated that both pachytene spermatocytes and round spermatids are capable of in vitro synthesis of plasma membrane proteins. In our assay at saturating levels there were only slightly larger numbers of bound round spermatids than pachytene spermatocytes on the Sertoli cell monolayers. We still are not able to mimic the in vitro ratio of pachytene spermatocytes to round spermatids bound per Sertoli cell. This may be due to the fact that the Sertoli cells used in our assay were isolated from immature animals; however, procedures for reproducibly isolating Sertoli cells from adult animals are not available, to our knowledge. We estimate that at most four to five pachytene spermatocytes and round spermatids bind per Sertoli cell in our in vitro assay. Perhaps a closer approximation to the situation in vivo may be achieved by using Sertoli cell monolayers cultured in bicameral chambers on extracellular matrix so that the epithelial cells assume a more columnar morphology.

In our competitive binding experiments pachytene spermatocytes were able to inhibit round spermatid binding, but round spermatids did not inhibit pachytene spermatocyte binding. This suggests that either the two cell types share some common binding site, with pachytene spermatocyte–Sertoli cell binding being of higher affinity than round spermatid–Sertoli cell binding, or that round spermatids bind a separate site, which is both of lower affinity and sterically hindered by the presence of bound pachytene spermatocytes. D’Agostino & Stefanini (1987) reported that an antiserum raised against rat pachytene spermatocytes, which inhibits their in vitro binding to Sertoli cell monolayers, retains its ability to inhibit pachytene spermatocyte binding even when preabsorbed against spermatids. In vivo, rat pachytene spermatocytes are bound to stage V Sertoli cell by one to four desmosome/gap junctions, with an average of 2–4 desmosome/gap junctions per pachytene spermatocyte (Russell et al. 1983; the magnification used for this ultrastructural study was not sufficient to distinguish between desmosomes and gap junctions). Step 5 round spermatids, on the other hand, form both desmosome/gap junctions and ectoplasmic junctional specializations with rat Sertoli cells (Russell et al. 1983). About half of the step 5 round spermatids examined had desmosome/gap junctions, and about one third had ectoplasmic junctional specializations. The desmosome/gap junctions that were present in round spermatids were much smaller (about 0.5 μm) than those present in pachytene spermatocytes (up to 1.2 μm). This may explain the lower slope of the round spermatid binding curve compared to that for pachytene spermatocytes. Russell (1980) believes that the ectoplasmic junctional specialization does not serve an adhesive function until the acrosomal membrane contacts the plasma membrane within the step 7 round spermatid. Our round spermatid fraction contains steps 1–8 round spermatids. As differentiation in vivo progresses, each spermatid becomes held to the Sertoli cell by one ectoplasmic junctional specialization per spermatid, and loses all desmosome-like junctions. Perhaps the initial adhesion events that ultimately develop into an ectoplasmic junctional specialization are much weaker than the initial adhesion events that ultimately develop into desmosome-like junctions. It is also possible that only a limited subset of our round spermatid fraction is capable of adhering to Sertoli cells; say, round spermatids of steps 7 and 8. Alternatively, the slightly higher number of round spermatid binding sites versus pachytene spermatocyte binding sites may thus be due to round spermatid binding to both desmosome-like recognition sites and ectoplasmic-junctional-specialization-like recognition sites, each with a different subset of round spermatids. The binding of pachytene spermatocytes to desmosome recognition sites might sterically inhibit round spermatid binding to both desmosome-like and ectoplasmic-junctional-specialization-like recognition sites on the Sertoli cells in vitro.

With this cell binding assay we cannot accurately determine the extent of non-specific binding as can be done for other receptor–ligand binding systems by adding 100-fold excess of unlabelled ligand, due to limited cell numbers available. Thus, an alternative explanation of the data could be that round spermatids bind with lower affinity to Sertoli cells and also undergo more non-specific binding than do pachytene spermatocytes.

Further differences between pachytene spermatocyte and round spermatid binding are evident from the ultrastructural observations. When mixed spermatogenic cell populations were added to the Sertoli cells monolayer and gently shaken for 60 min, then fixed immediately with paraformaldehyde and glutaraldehyde and processed for electron microscopy, very few spermatogenic cells adhere to the monolayer (unillustrated observations). When adhering spermatogenic cells are cocultured for 24–48 h and then fixed and processed for electron microscopy, spermatocytes are observed adhering to Sertoli cells by structures similar
to desmosome-like junctions, which are present in vitro. However, round spermatids apparently do not form junctions stable enough to be retained through culture and processing for electron microscopy. The round spermatids' lack of ability to form stable junctions may be due to a more limited ability of ectoplasmic junctional specialization sites to form stable adherent junctions in vitro, or that desmosome-like junction adhesion mechanisms normally disappear rather than form at this stage in development. Ultimately the use of our assay system in conjunction with inhibitory antisera that could specifically inhibit the binding of a given stage of spermatogenic cells should be able to answer some of the questions raised by our results.

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