

Alkaline Phosphatase Histochemistry Discriminates Peritubular Cells in Primary Rat Testicular Cell Culture

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Histochemical demonstration of alkaline phosphatase activity appears to be useful in identifying rat peritubular cells in primary testicular cell culture. In both frozen sections of rat testis and Mirsky's fixed, methacrylate-embedded rat testis, the reaction product localized primarily in peritubular cells, vascular endothelium and occasionally in interstitial cells, with much smaller amounts of reaction product associated with elongating spermatids in the germinal epithelium. Occasional late-stage tubules (X-XIV) showed weak reactivity in the epithelium, associated with spermatocytes or Sertoli cells. Ultrastructurally, Gomori-method reaction product was localized to peritubular cells, lymphatics, and spermatogonia in stage VII; no staining was found consistently in Sertoli cells. In isolated cell preparations enriched for Sertoli and germ cells, 1 to 8% of the cells demonstrated alkaline phosphatase activity, while greater than 50% of the cells stained positive for alkaline phosphatase activity in peritubular-enriched fractions. The histochemical demonstration of alkaline phosphatase activity can be useful for identifying peritubular cells in primary cultures of testicular cells.

Key words: peritubular cells, alkaline phosphatase, histochemistry, Oil Red O.

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Primary cultures of testicular cells are being used increasingly in physiologic and toxicologic studies. Before interpreting the data from these studies, it is necessary to define the population of cells being analyzed. In Sertoli cell-enriched cultures, one of the major challenges is to distinguish between the Sertoli cells and the peritubular myoid cells. At the level of the phase contrast light microscope, these cells appear similar. Indeed, "the lack of good biochemical or histochemical markers for myoid cells makes quantification of this cell type in culture particularly difficult" (Mather and Phillips, 1984a). In characterizing our rat Sertoli cell-enriched cultures for toxicologic studies, we examined histochemical stains for several enzymes and describe in this report data that support the use of alkaline phosphatase as a histochemical marker for peritubular cells in culture. The data come from examining alkaline phosphatase activity in the following preparations described in this order: frozen and fixed sections of testis at the

level of the light microscope, fixed testis sections at the electron microscopic level, fragments of seminiferous tubules stained during the isolation process, and enriched isolated cell populations cultured for varying lengths of time.

Materials and Methods

Animals

Male and female Fischer 344 rats (CDF [F344]/CrI/BR, 200–250 g) were obtained from the Kingston, NY colony of Charles River Laboratories. Animals were acclimated to the NIEHS colony for at least 2 weeks prior to use. Animals were housed three/polycarbonate cage with hardwood bedding, with an *ad libitum* supply of food (NIH-07 pellets) and filtered tap water, lights on 0800 to 2000 hours, humidity $50 \pm 10\%$, and ambient temperature 20 ± 2 C. Young rats for the cell separation studies were born of F344 parents in the NIEHS colony, housed under identical conditions as above, and sacrificed on day 18 *post-partum*. Animals for frozen sections and cell separation studies were sacrificed by asphyxiation with CO₂.

All chemicals and hormones were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Tissue Preparation

Testes for frozen sections were frozen to brass cryostat chucks on dry ice, and cut at 6 μm on a Harris cryostat; sections were stored at -20 C until assayed.

Adult rats to be perfused for fixed sections (light microscopy) were deeply anesthetized with methoxyflurane (Pitman-Moore, Washington Crossing, NJ), and perfused through the ascending aorta with Ringer's balanced salts containing 0.1% procaine and 0.01% sodium nitroprusside. When the testicular veins were clear, the animals were perfused with Mirsky's fixative (National Diagnostics, Sommerville, NJ) for 5 minutes at room temperature. The testes were removed, put in Mirsky's on ice, and kept at 4 C for the remaining processing steps. A cross section of the testis was cut (2-mm thick), rinsed in 0.1 M sodium phosphate (pH 7.4) for 30 minutes, put in one change of uncatalyzed Immunobed[®] methacrylate (Polysciences, Warrington, PA) diluted 1:1 with glass distilled water for 3 hours on a rotator in a 4 C cold room, then passed through two changes (for 3 hours and 16 hours, respectively) of undiluted uncatalyzed Immunobed[®] with rotation, and embedded in Immunobed[®] at 4 C.

Methacrylate-embedded sections were cut with a standard D profile steel knife on a Leitz 1512 microtome set at 2 μm , mounted on room temperature slides, and stored at 4 C until assayed.

Tissues for the ultrastructural localization of alkaline phosphatase activity were perfused as above, but fixed with 10% neutral buffered formaldehyde. After 4 hours fixation, a 2-mm-thick slice of testis was diced into blocks $2 \times 2 \times 2$ mm, and rinsed three times with 0.1 M phosphate buffer, pH 7.4. The alkaline phosphatase reaction was carried out at room temperature (Gomori, 1941), after which the tissue was then processed into Epon, and examined on a Philips 400 electron microscope.

Enriched Cell Preparations

Enriched cell preparations were prepared by the method of Kierszenbaum and Tres (1981). Eighteen-day-old rats were asphyxiated with CO₂, and each testis was decapsulated and minced in Hanks' balanced salt solution. The cell clumps were separated from individual cells by unit gravity, and incubated in 0.4% w/v trypsin (Sigma cat. no. T-0511) in Hanks', with DNase (0.5 mg/50 ml Hanks') at 32 C for 30 minutes in a shaking waterbath (Fisher model 125, 90 cycles/minute). Up to 56 testes were incubated per 50 ml of Hanks'-trypsin-DNase. At the end of the incubation, the cells were aspirated through a 10-ml sterile pipet ten times, the tubular fragments were allowed to settle for 3 minutes at unit gravity, and then the supernatant was discarded. The tubular fragment pellet was resuspended in collagenase (Sigma no. C-5894, 0.1% in 35 ml Hanks' with 0.5 mg DNase) and incubated as above for 60 minutes. After incubation, the tissue was aspirated as above and the tubular fragments were allowed to settle at unit gravity for 3 minutes. The supernatant contained mostly peritubular cells and some small tubule fragments and was plated out for studies of peritubular-enriched cultures. For Sertoli-enriched cultures, the supernatant was discarded, and the pellet was washed with 35 ml of 0.01% soy trypsin inhibitor in Minimal Essential Medium with Earle's salts (MEM). The pelleted cells were resuspended in MEM and counted by hemocytometer. For histochemistry, cells were plated on untreated polystyrene plates (Integrid[®] petri plates, Falcon #1012, 100 cm²) at subconfluent densities. The following morning, the medium containing unattached cells was washed off and replaced with MEM containing transferrin (5 $\mu\text{g}/\text{ml}$), retinol acetate (5 μM), epidermal growth factor (3 ng/ml), bovine insulin (5 $\mu\text{g}/\text{ml}$), testosterone and dihydrotestosterone (0.1 μM each), and growth hormone (6.5 $\mu\text{U}/\text{ml}$) (Tres and Kierszenbaum, 1983). No antibiotics or serum were used in the isolation or maintenance phase of the culture. Cells were maintained at 32 C in 5% CO₂; the hormone-supplemented medium was changed every other day. After a variable period of time *in vitro* (1–8 days), plated cells were fixed with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 minutes at room temperature prior to staining.

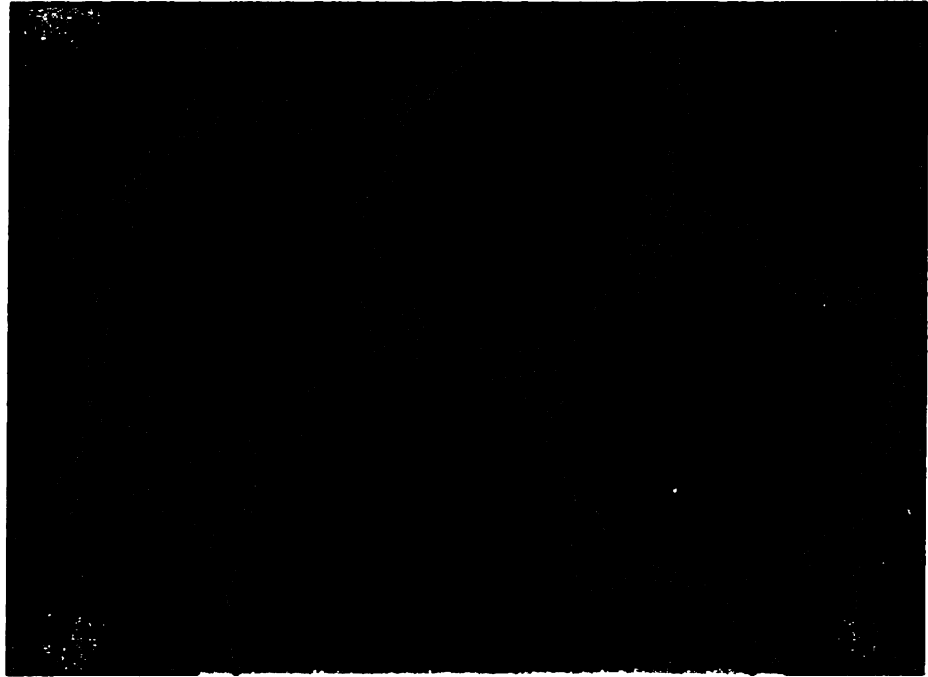
For histochemical demonstration of alkaline phosphatase activity on unplated tubule fragments, an aliquot of the final cell preparation was removed just prior to plating and mixed gently with an equal volume of 8% buffered formaldehyde for 5 minutes. The cells were then pelleted by centrifugation and resuspended in amino-methyl-propanol buffer (below). This rinse was repeated twice. The pellet was then resuspended in the reaction mixture for the alkaline phosphatase assay (below).

Histochemistry

The reaction is a simultaneous coupled azo dye method, using a polysubstituted naphthol as substrate. An excellent discussion of these techniques is found in Pearse (1968). The naphthol produced by the reaction is joined with a diazonium salt to form a water-insoluble colored compound that deposits on the surrounding protein.

The reaction buffer was 0.25 M 2-amino-2-methyl-1-

Fig. 1. Section of frozen testis, stained for alkaline phosphatase coupled to Fast Blue RR, counterstained with Nuclear Fast Red. The dark deposits indicating enzyme activity (arrowheads) are limited to selected interstitial cells and peritubular elements ($\times 250$).



propanol, containing 1.25 mM $MgCl_2$ (pH 8.9). This buffer has been found to enhance alkaline phosphatase activity (Reasor et al, 1978). The reaction is that described by Miller et al (1987) and was prepared by dissolving 50 mg of substrate (Naphthol AS-BI phosphoric acid) in 200 μ l dimethyl sulfoxide. Twenty-five milliliters of double-distilled water and 25 ml of amino-methyl-propanol buffer were then added and mixed well. Immediately prior to use, 50 mg of either Fast Red Violet LB or Fast Blue RR salt were added to this mixture, mixed vigorously, and filtered. The reaction mixture was incubated at 22 C for 15 minutes on tissue sections, and for 10 minutes on plated cells and tubule fragments. The reaction mixture was then removed, and the sections or cells were rinsed twice with amino-methyl-propanol buffer. Tissue sections were counterstained with either hematoxylin or Nuclear Fast Red.

Tubule fragments were pelleted at the end of the reaction time, and rinsed twice with amino-methyl-propanol buffer. Fragments were not counterstained, but resuspended in a small amount of the buffer, dropped on a slide, cover-slipped, and examined on a Nikon Optiphot microscope with a UFX camera.

After staining for alkaline phosphatase, plated cells were rinsed with 100% propylene glycol, and stained for 45 minutes at 32 C with 0.5% (w/v in propylene glycol) Oil Red O to stain neutral lipid droplets in Sertoli cells (Luna, 1968). After staining, cells were rinsed once with 85% propylene glycol, and then twice with amino-methyl-propanol buffer. For plated cells, we routinely used Fast Blue RR and Oil Red O to determine if cells stained for both lipid and alkaline phosphatase. The plates were examined on an inverted phase contrast microscope (Leitz Diavert), and were then cover-slipped with an aqueous mounting medium and stored at room temperature.

As an alternative method, which does not identify lipid

droplets but does stain nuclei, the alkaline phosphatase reaction can be coupled to Fast Red Violet LB. The cells then can be stained with hematoxylin for 3 minutes and washed with water for 5 minutes. The hematoxylin can be "blued" with 0.5% sodium carbonate or calcium carbonate for 15 seconds, and mounted with an aqueous mounting medium. This produces blue nuclei and red alkaline phosphatase reaction product.

To assess the stability of alkaline phosphatase activity in the cultures over time, five plates of cells were prepared in some experiments, and one plate was fixed with formaldehyde daily on days 3 to 8, and assayed for alkaline phosphatase activity as above. At least 1000 total cells/plate, or eight randomly selected visual fields, were counted, whichever was greater. In another experiment, eight plates of cells were prepared, and four plates each were fixed and stained on days 3 and 7 after plating.

Results

Figure 1 shows that alkaline phosphatase activity in a frozen section of rat testis was strongest in the peritubular compartment and in occasional interstitial cells. There was also strong reactivity in the vascular endothelium (not shown). Figure 2 shows a section from a Mirsky's fixed, GMA-embedded rat testis, which confirmed the findings of the frozen section and also showed some weak diazonium reaction product inside the tubular epithelium. At this level, it was not possible to associate confidently this faint staining with a specific cell type. Figure 3 shows that, at the ultrastructural level, the cobalt ions from the Gomori reaction deposited in the cytoplasm and

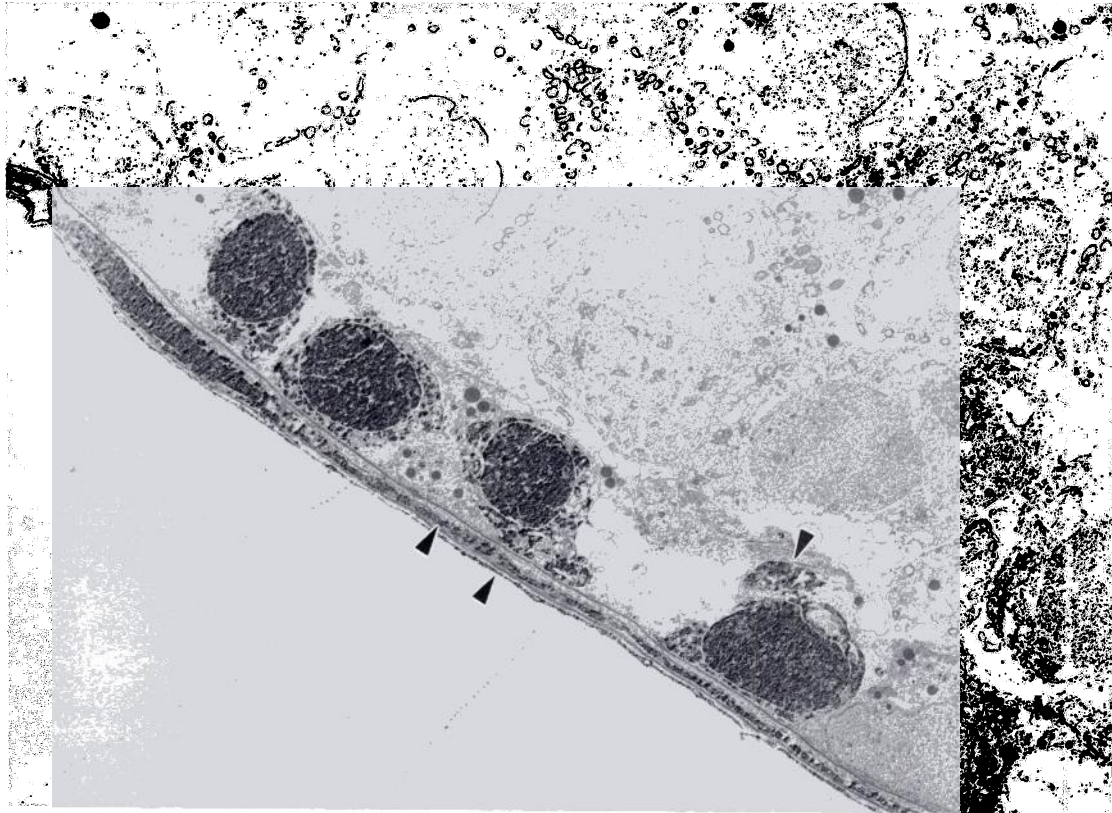


Fig. 3. Electron micrograph of Gomori reaction deposits in a stage VII seminiferous tubule. There is significant stain deposition (arrowheads) in the peritubular, lymphatic, and spermatogonial elements, and little discernible Sertoli cell staining ($\times 2470$).

nucleus of peritubular cells and some spermatogonia; there was no discernible reaction product in other cells of the epithelium. The nuclear deposition of heavy metal reaction product is a common artifact of the Gomori method (Pearse, 1968). There was faint reaction product associated with testicular spermatozoa in the lumen of both seminiferous and epididymal tubules (not shown).

Figure 4 shows some tubule fragments stained for alkaline phosphatase before plating. Although not seen clearly in this figure, sections of tubules circumscribed by alkaline phosphatase-positive cells appeared constricted compared with those areas of tubules without such cells.

Figure 5 shows the appearance of cells plated for 3 days prior to fixation and staining. The alkaline phosphatase reaction product in both uncultured fragments (Fig. 4) and in plated cells (Fig. 5) appears as a granular deposit on a reticular perinuclear network. In our Sertoli-enriched preparations, alkaline phosphatase-positive cells comprise 1% to 8% of the total population. Of the $> 40,000$ total cells counted to date, 13 (0.039%) have stained for both alkaline

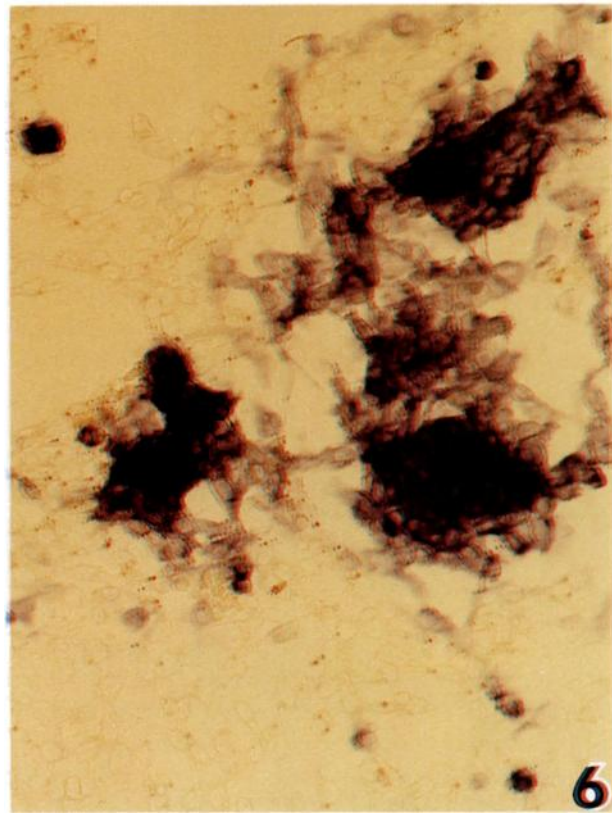
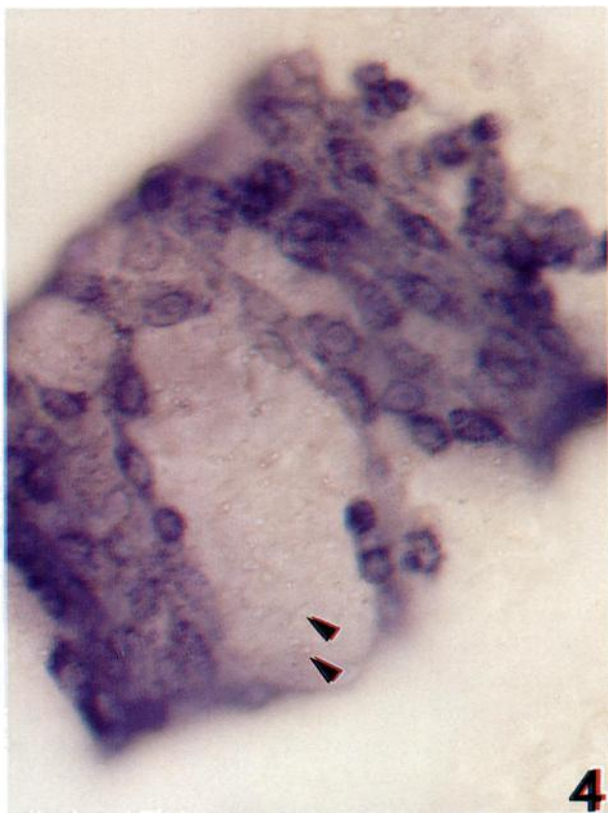
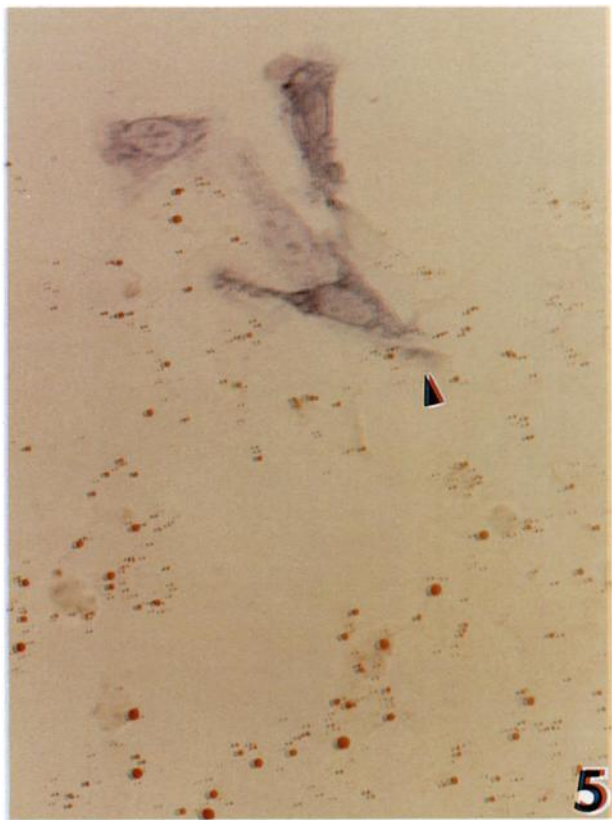
phosphatase and neutral lipid. Cells that stain for both have a fainter and more diffuse staining that is

Section of testis fixed with Mirsky's fixative, embedded in glycol methacrylate. The alkaline phosphatase reaction is coupled to Fast Red Violet LB, counterstained with hematoxylin. Note that while the majority of activity appears around and outside the tubules (arrows), there is faint stain deposition inside the tubules (arrowheads) ($\times 500$).

Fragment of seminiferous tubule after sequential incubation with trypsin and collagenase. The alkaline phosphatase reaction was coupled to Fast Blue RR salt, producing a blue precipitate. Note the granular stain deposition concentrated near the nuclei, that the deposits appear darker in some cells than in others, and the bright lipid droplets (arrowheads) in areas that lack peritubular cells ($\times 500$).

Cell fraction enriched for Sertoli cells (arrows), plated 3 days, fixed, and stained for lipid with Oil Red O and for alkaline phosphatase, coupled with Fast Blue RR. Note that stain deposition pattern is similar to those cells in Fig. 4; also the slight product deposition on the edges of the fat-stained cells (arrowheads) ($\times 500$).

Peritubular-enriched fraction, plated 3 days, fixed, and stained for neutral lipid with Oil Red O and for AP, coupled to Fast Blue RR. The relative increase in numbers of peritubular cells is characteristic of this fraction ($\times 250$).



not concentrated around the nucleus; these are almost always seen closely apposed to several cells showing intense alkaline phosphatase activity.

The peritubular-enriched fraction is shown in Fig. 6 stained 3 days after plating. Routinely, greater than 50% of the cells in this fraction showed stain deposition in a pattern also seen in the alkaline phosphatase-positive cells in Fig. 4. This fraction also contained elongated spindle-shaped cells that stained for neither lipid nor alkaline phosphatase activity.

In Sertoli cell-enriched cultures, the percentage of cells that were alkaline phosphatase-positive in four plates was similar at 3 and 7 days post-plating; the values (mean + SD) for these groups were $4.71\% \pm 1.91\%$ and $5.58\% \pm 1.52\%$, respectively. The group means were not statistically different by student's *t* test, which was confirmed by analyzing individual plates at different times up to 9 days post-plating. In the three experiments in which this was done, there was no upward trend in the percentage of cells that were alkaline phosphatase-positive (data not shown).

Discussion

The data presented above indicate that the major portion of alkaline phosphatase activity in the testis is localized in peritubular cells and interstitial elements, while little or no activity is found in the germinal epithelium itself. This localization of activity has been reported previously at both the light and electron microscopic levels (Tice and Barnett, 1963; Kormano, 1967; El-Maghraby and Gardner, 1968; Kormano and Hovatta, 1974; Kanwar et al, 1974; Redi et al, 1983). In contrast to these authors, we observed a very slight amount of epithelial staining in both the Mirsky's-fixed and frozen sections of the testis that could be associated with either Sertoli cells or germ cells. The ultrastructural sections showed no consistent pattern of activity other than that seen in some spermatogonia and in all peritubular cells. However, the presence of slight activity in elongating spermatids and epididymal spermatozoa is consistent with the localization of the faint epithelial alkaline phosphatase activity to these abundant germ cells. In any case, it is clear that by far the greatest activity is found in the peritubular elements.

Kornblatt et al (1983) used biochemical and developmental studies to localize alkaline phosphatase activity to the nongerminal cells of the epithelium and concluded that Sertoli cells were the site of this activity. This finding is at variance with the weight of the evidence in the literature, and is likely due to the fact that they did not separate peritubular cells from

the Sertoli cells and germ cells in their preparation. The status of alkaline phosphatase in germ cells is unresolved, reported as being either present or absent in spermatozoa of the rat (Terner et al, 1975, and Moniem and Glover, 1972, respectively).

The *in vitro* data are consistent with the results from tissue sections. The trypsin-collagenase treatment above has been found to remove most peritubular cells, producing a Sertoli-enriched population (Kierszenbaum and Tres, 1981). Thus, plating the peritubular-enriched fraction yields a culture where 50% or more of the cells stain positive for alkaline phosphatase (Fig. 6). Additionally, these cells show a stain pattern and distribution similar to that seen in cells attached to tubule fragments. Not surprisingly, when cells are plated at confluent densities, the alkaline phosphatase-positive cells most often are found between the Sertoli cells and the plastic substrate. Due to the diffusion of the reaction product, or a less likely exchange of membrane components, the overlying cells frequently appear to contain alkaline phosphatase activity. Therefore, cells for histochemistry are seeded at one-tenth to one-twentieth the usual confluent density. Plated in this way, the alkaline phosphatase-positive cells frequently migrate away from tubular fragments, and are easily distinguished. In addition, neutral lipid staining with Oil Red O is used to facilitate the differentiation of Sertoli cells. We have noted that in some preparations, up to about 2% of the "Sertoli" cells do not contain lipid droplets under these culture conditions; the reasons for this occasional focal change are still unclear.

We have noticed that occasional germ cells reside atop alkaline phosphatase-positive cells in culture; this is not inconsistent with the placement of spermatogonia *in vivo* between the myoid cells and the overlying Sertoli cells. Frequently, spermatogonia will show alkaline phosphatase activity *in vitro*, which is also congruent with *in vivo* findings (Redi et al, 1983). These cells appear spherical and lack the spreading attachment points seen with peritubular and Sertoli cells.

The fact that the percent of cells that are alkaline phosphatase-positive does not increase with time in culture supports the concept that the Sertoli cells do not express alkaline phosphatase activity with time *in vitro*, at least in this limited time frame. We have not examined the activity over longer periods or in established cell lines. Thus, while Sertoli cells do acquire β -adrenergic receptor activity *in vitro* that they lack *in vivo* (Kierszenbaum et al, 1985), this does not seem to be true of alkaline phosphatase activity.

Cooperativity between peritubular cells and Sertoli cells has been shown to occur both *in vivo* (Kanwar et al, 1974) and *in vitro* (Mather and Phillips, 1984b; Skinner and Fritz, 1985; Skinner et al, 1985; Cameron and Snyder, 1985). For both cooperativity studies and those on isolated, purified cell types, we feel that alkaline phosphatase histochemistry, either alone or in conjunction with lipid staining, is a useful tool to help define the types of cells present in the culture.

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References

- Cameron DF, Snyder E. Selected enzyme histochemistry of Sertoli cells. 2. Adult rat Sertoli cells in co-culture with peritubular fibroblasts. *Andrologia* 1985; 17:185-193.
- El-Maghraby MAHA, Gardner DL. A comparative study in young male animals of 10 species of the distribution of alkaline phosphatase activity in small arteries. *Histochemie* 1968; 16:227-235.
- Gomori G. The distribution of phosphatase in normal organs and tissues. *J Cell Comp Physiol* 1941; 17:71-84.
- Kanwar KC, Bawa SR, Singal PK. Testicular hyperthermic shocks and the boundary tissue of the seminiferous tubules in rats. *J Reprod Fertil* 1974; 41:201-204.
- Kierszenbaum AL, Spruill WA, White MG, Tres LL, Perkins JP. Rat Sertoli cells acquire a β -adrenergic response during primary culture. *Proc Natl Acad Sci USA* 1985; 82:2049-2053.
- Kierszenbaum AL, Tres LL. The structural and functional cycle of Sertoli cells in culture. In: Jagiello G, Vogel HJ, eds. *Bioregulators of reproduction*. New York: Academic Press, 1981; 207-228.
- Kormano M. Dye permeability and alkaline phosphatase activity of testicular capillaries in the postnatal rat. *Histochemie* 1967; 9:327-338.
- Kormano M, Hovatta O. Intense phosphatase activity in the developing rete testis of the newborn rat. *Histochemistry* 1974; 49:99-104.
- Kornblatt MJ, Klugerman A, Nagy F. Characterization and localization of alkaline phosphatase activity in rat testes. *Biol Reprod* 1983; 29:157-164.
- Luna LG. *Manual of histologic staining methods of the Armed Forces Institute of Pathology*; Third Edition. New York: McGraw-Hill, 1968; 140-141.
- Mather JP, Phillips DM. Primary culture of testicular somatic cells. In: Barnes D, Sirbasku D, Sato G, eds. *Methods of serum-free culture of cells of the endocrine system*. New York: Alan R. Liss, 1984a; 29-45.
- Mather JP, Phillips DM. Establishment of a peritubular myoid-like cell line and interactions between established testicular cell lines in culture. *J Ultrastruct Res* 1984b; 87:263-274.
- Miller BE, Chapin RE, Pinkerton KE, Gilmore LB, Maronpot RR, Hook GER. Quantitation of silica-reduced Type II cell hyperplasia by using alkaline phosphatase histochemistry in glycol methacrylate embedded lung. *Exper Lung Res* 1987; 12:135-148.
- Moneim KA, Glover TD. Alkaline phosphatase in the cytoplasmic droplet of mammalian spermatozoa. *J Reprod Fertil* 1972; 29:65-69.
- Pearse AGE. Alkaline phosphatases. In: *Histochemistry: theoretical and applied*. Boston: Little, Brown & Company, 1968; 495-546.
- Reasor MJ, Nadeau D, Hook GER. Extracellular alkaline phosphatase in the airways of the rabbit lung. *Lung* 1978; 155:321-335.
- Redi CA, Hilscher B, Winking H. Stage-dependent enzymatic activities in spermatogenesis of mice with the standard karyotype and of chromosomal variants with impaired fertility. *Andrologia* 1983; 15:322-330.
- Skinner MK, Fritz IB. Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions. *Proc Natl Acad Sci USA* 1985; 82:114-118.
- Skinner MK, Tung PS, Fritz IB. Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. *J Cell Biol* 1985; 100:1941-1947.
- Terner C, MacLaughlin J, Smith BR. Changes in lipase and phosphatase activities of rat spermatozoa in transit from the caput to the cauda epididymidis. *J Reprod Fertil* 1975; 45:1-8.
- Tice LW, Barnett RJ. The fine structural localization of some testicular phosphatases. *Anat Rec* 1963; 147:43-63.
- Tres LL, Kierszenbaum AL. Viability of rat spermatogenic cells in vitro is facilitated by their coculture with Sertoli cells in serum-free hormone-supplemented medium. *Proc Natl Acad Sci USA* 1983; 80:3377-3381.