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Alkaline Phosphatase Is a Marker for Myoid Cells in Cultures of Rat Peritubular and Tubular Tissue[1]

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ABSTRACT

We have studied the distribution of histochemically detectable alkaline phosphatase in cultures of seminiferous tubule fragments and of peritubular cells from prepubertal rats. The same material also was immunohistochemically evaluated for the presence of desmin-containing intermediate filaments. The comparative analysis of alkaline phosphatase and desmin positivity shows that alkaline phosphatase histochemistry selectively detects desmin-containing contractile cells in tubular and peritubular cell cultures. We propose alkaline phosphatase as a novel marker for myoid cells that can be of help in screening, defining, and eventually standardizing the exact composition of peritubular cell cultures, a model that is of increasing interest in the study of cellular interactions in the testis.

INTRODUCTION

The term "peritubular cells" defines a heterogeneous cellular population located at the boundary between seminiferous epithelium and interstitium. In the rat testis, this population is composed of an inner layer of contractile myoid cells and an outer, thin and discontinuous layer of lymphatic endothelium (Leeson and Leeson, 1963; Dym and Fawcett, 1970). Peritubular cells have received renewed interest since they have been shown (Skinner and Fritz, 1985; Fritz and Tung, 1986, for a review) to be capable of functional interaction in vitro with Sertoli cells, the somatic component of seminiferous epithelium. Such interaction appears to be under testosterone control (Skinner and Fritz, 1985), and now it seems more likely than in the past that peritubular cells may be involved in the regulation of spermatogenesis. So far, most of the information on peritubular cell products and behavior has been derived from primary or secondary cultures of a highly heterogeneous cell population obtained through enzymatic digestion of the tubular wall. Besides myoid cells, lymphatic endothelial cells and interstitial fibroblasts are probably present within this ill-defined population, in ratios presumably varying with isolation and culturing protocols. For the identification of the individual cell types within the bulk population and further characterization of their functions, specific cell markers are needed.

Quite recently (Virtanen et al., 1986), by means of a monoclonal antibody, desmin-containing intermediate filaments have been shown to be present in the myoid cell, thus providing a useful marker for this cell type. We show here that alkaline phosphatase, an enzyme that has been shown to be present in rat peritubular tissue in vivo (Santiemma et al., 1978) and in vitro (Chapin et al., 1987) and that represents a well-known marker for myoepithelial basket cells of the mammary gland secretory units (Soloff et al., 1980), is a marker for desmin-containing myoid cells in cultures of peritubular cells and of tubular material.

MATERIALS AND METHODS

Experimental Animals

For all experiments, the testes were excised from 3-wk-old prepubertal Wistar rats killed by cervical dislocation.

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Preparation of Cryosectioned Testis Samples

Pieces of testes were frozen in freon precooled in liquid nitrogen, and 6-μm sections were cut in a Leitz cryostat (Leitz Wetzlar GMBH, Wetzlar, W. Germany). After mounting on gelatine-coated slides and drying at room temperature, the sections were fixed in cold acetone/ethanol fixative (1:1 v/v) for 10 min at −20°C, then transferred to 0.1 M phosphate-buffered saline (PBS).

Preparation of In Vitro Cultures

The procedure followed for peritubular cell cultures is, in principle, that indicated by Tung and Fritz (1977). In brief, testicular fragments were subjected to enzymatic digestion, first with trypsin to remove the interstitium, then with collagenase to detach peritubular cells; after sedimentation of the tubular fragments, the supernatant was centrifuged at 40 × *g* for 10 min; the pellet thus obtained was composed of minute fragments of seminiferous tubule wall and was plated to yield peritubular cells in coculture with Sertoli cells. The supernatant, mainly composed of single cells, was centrifuged again at 200 × *g* for 10 min, and the resulting pellet was plated as peritubular cell preparation.

Culture Conditions

The minute fragments of tubular wall were cultured 1–4 days in plastic dishes at 32°C in Eagle's Minimum Essential Medium (MEM) in a humidified atmosphere containing 5% CO_2. For peritubular cell suspensions, the culture medium was supplemented with 10% fetal calf serum and the incubation was performed at 37°C. For double staining, the cells were plated on glass tissue culture chamber slides (Lab Tek, Miles Laboratories Inc., Naperville, IL) or plastic slide flasks (Nunc, Roskilde, Denmark), both of which allow repeated identification of single cells in the microscope. As a fixative for the cultured material, cold acetone/ethanol was used as described for cryosections for all kinds of further processing.

Histochemical Localization of Alkaline Phosphatase Activity

The method of Ackermann (1962) was essentially followed; sections and cultured material were incubated in the dark for 30 min in an alkaline solution (pH 8.6) containing 0.5 mg/ml Fast Blue RR (Sigma Chemical Co., St. Louis, MO) in water and 40 μl/ml α-napthol-phosphate (0.25% solution, Sigma). After rinsing in water, the samples were mounted in Aquamount (BDH Chemicals, St. Laurent, Quebec, Canada).

Immunocytochemistry

The presence of desmin-containing intermediate filaments was checked with immunofluorescence. Before starting the immunolocalization procedure, the fixed specimens were incubated for 1 h in 5% Carnation nonfat dry milk (Carnation Co., Los Angeles, CA) in PBS to minimize nonspecific antibody binding (Johnson et al., 1984). The primary antibody (used at 1:5 dilution for 1 h) was a monoclonal anti-desmin antibody (Amersham International, Amersham, UK) raised against porcine stomach desmin. After thorough washing in PBS, the mouse antibodies were labeled with a fluorescein isothiocyanate-coupled rabbit anti-mouse antiserum (Zymed Laboratories Inc., San Francisco, CA). The specificity of the reaction was checked in control samples prepared with the primary antibody omitted. Both slides and culture dishes were mounted in glycerol.

Double Staining

With cryosections, immunostaining could be followed by histochemical processing for alkaline phosphatase, with only minimal reduction in fluorescence. For cultured cells, a different approach had to be used, since the presence of the enzymatic reaction product on the cell membrane was found to interfere with fluorescence. To circumvent this problem, the cells, which had been cultured on slides (see above), were immunostained. Selected fields were photographed, with the exact position of the slide on the microscope stage (equipped with micrometric graduated scales) noted. Coverslip and glycerol then were gently removed by immersion in PBS, and the sample was processed for alkaline phosphatase chemistry. Finally, the previous microscopic fields were traced and photographed again. The microscope used was a Leitz Orthoplan, equipped with Vario-Orthomat photographic equipment.

RESULTS

Testis Cryosections

Figures 1 and 2 allow comparison of the distribution of anti-desmin antibody and of alkaline phos-

phatase activity in the testes of 3-wk-old rats. As expected, desmin-containing intermediate filaments were present in peritubular cells as well as in smooth muscle cells of arteriolar walls. On the other hand, alkaline phosphatase positivity was observed in the peritubulum and on arteriolar and capillary endothelium, and was absent from any cell type within the seminiferous epithelium. Light microscopy resolution in cryosections did not allow assessment of whether other cell type(s) within the peritubulum were recognized by the enzymatic marker.

Tubular Wall Explants

After adhesion of the fragments to the substrate, peritubular cells formed patches of monolayer in which both desmin and alkaline phosphatase positivity were present (Fig. 3). Desmin-negative cells present in the culture, mainly Sertoli cells, appeared negative for alkaline phosphatase.

Peritubular Cell Cultures

In primary cultures of collagenase-dispersed peritubular cells, desmin-positive myoid cells (Fig. 4)

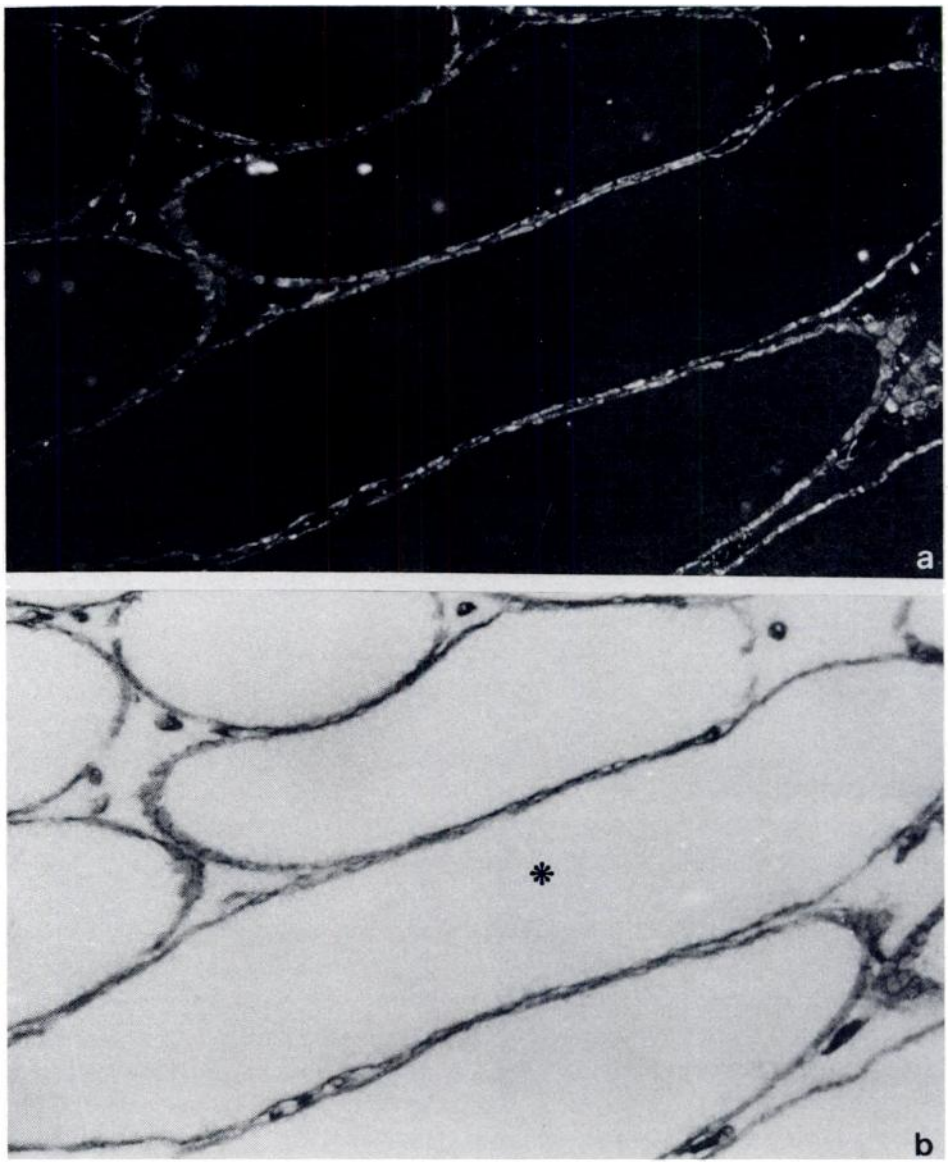

FIG. 1. Cryosection of 20-day-old rat testis immunostained for desmin (*a*) and subsequently processed for the detection of alkaline phosphatase activity (*b*). The peritubular tissue appears positive for both markers, whereas the seminiferous epithelium (*asterisk*) appears negative. ×520.

appeared to represent a major but far from exclusive component; their actual percentage in our short-term primary cultures of peritubular cells was in fact found to vary from 30% to 50%. As shown in Figures 5 and 6, alkaline phosphatase activity is detected in the same cells recognized by anti-desmin antibodies. An interesting observation of cellular shape was made during the first day after plating; at this culture time, peritubular cells appeared to be heterogeneous in shape and, in general, both markers seemed to be absent from the spindle-shaped and very elongated cells. Alkaline phosphatase positivity revealed the difference in shape between myoid and other cell types more dramatically, exhibiting (Figs. 5 and 6) a wide halo of peripheral phase-lucent cytoplasm, which is partly negative to desmin. This particular morphology of the myoid cell was progressively lost during the following days, when different cell types could no longer be detected on the basis of shape differences (not shown).

DISCUSSION

The presence of desmin-containing cells as the major component of the rat peritubulum has been recently demonstrated by Virtanen et al. (1986), by means of monoclonal and polyclonal antibodies. In the same year, Kierszenbaum et al. (1986), using differently prepared cells and antibodies, found peritubular cells to be negative for desmin. Our results, obtained with a commercial antibody raised against porcine stomach desmin, confirm the presence of desmin-containing intermediate filaments in myoid cells, in vivo (Figs. 1 and 2) as well as in culture (Figs. 3 to 6).

Histochemical positivity for alkaline phosphatase in the rat testis has long been known (Kormano, 1967; Santiemma et al., 1978; Kormano and Hovatta, 1974; Niemi and Setchell, 1986) and, recently, the testicular enzyme has also been biochemically characterized (Kornblatt et al., 1983). However, due to

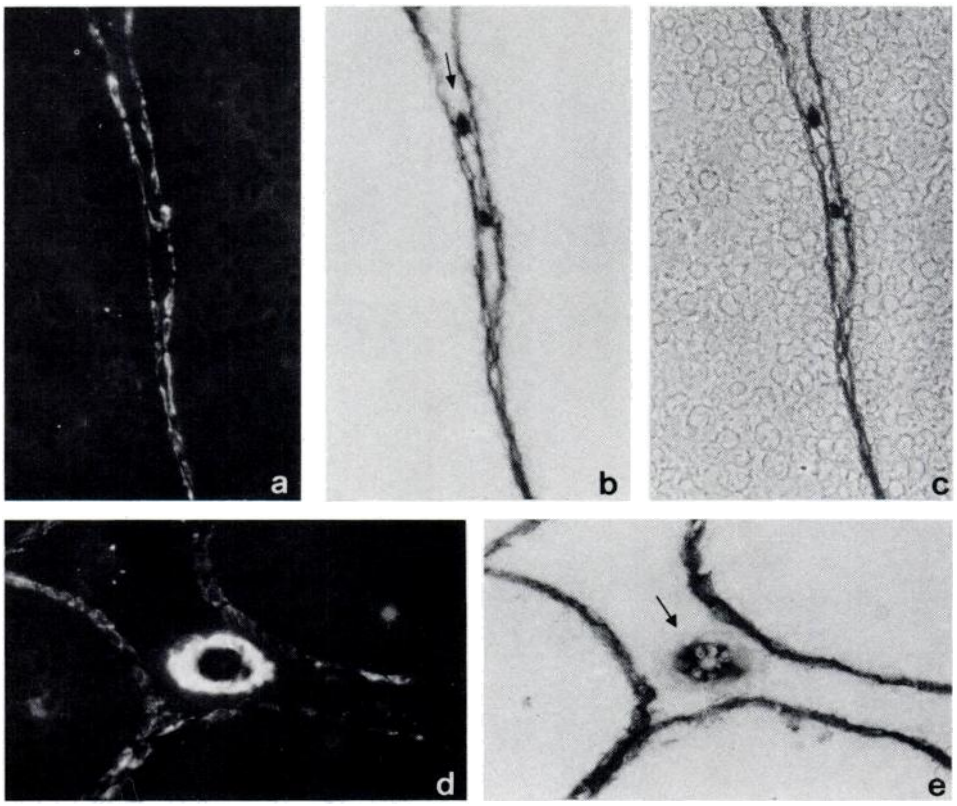

FIG. 2. Same material as in *Figure 1* at higher magnification to show the distribution of the two markers in the seminiferous tubule and in interstitial vascular structures; the endothelia of blood capillaries (*b*) and of arterioles (*e*) appear positive for alkaline phosphatase (*arrows*), whereas the smooth muscle cells of the arterioles are positive for desmin (*d*). Germ cells, apparent in phase-contrast microscopy (*c*), are negative for both markers. *a, b, e,* ×870; *d, e,* ×790.

contrasting results and/or interpretations of the histochemical data, its exact localization has remained an open subject. In the fetal testis, it is well established that alkaline phosphatase is a marker for primordial germ cells (McAlpine, 1955), but controversial data have been produced on the positivity of germ cells in more advanced developmental stages (see references in Chapin et al., 1987). Since alkaline phosphatase is a ubiquitous enzyme (Kornblatt et al., 1983), contrasting results can be obtained by use of histochemical methods of different sensitivity. In accordance with previous reports (Kormano, 1967; Santiemma et al., 1978; Niemi and Setchell, 1986; Chapin et al., 1987), and presumably because of quantitative differences, the approach we currently use exclusively detects alkaline phosphatase activity in the peri-

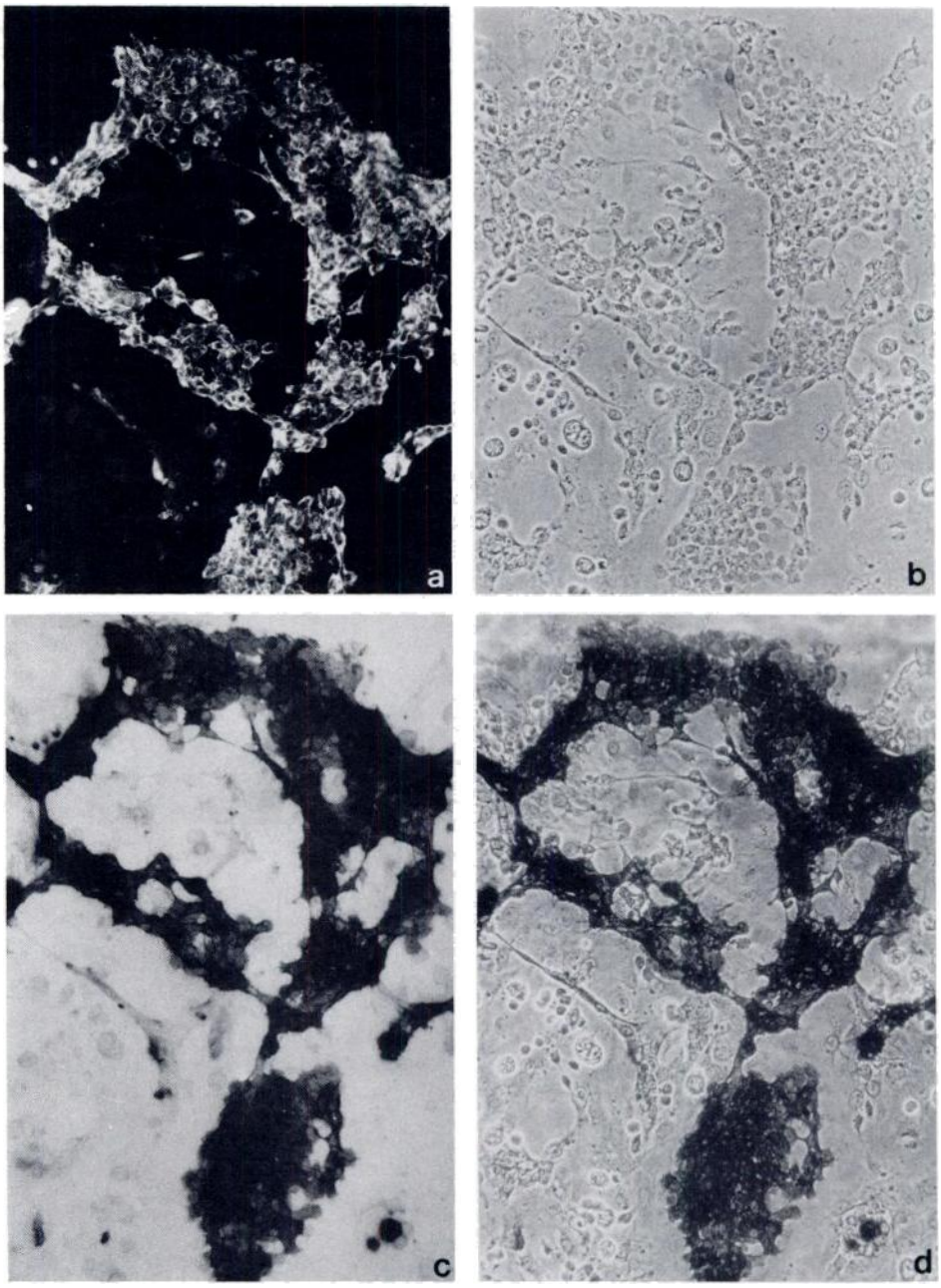

FIG. 3. Small fragments of tubular wall in culture for 3 days at 32°C on glass chamber slide. (*a*) and (*b*) Photographed after immunostaining for desmin (*b*, phase-contrast optics); (*c*) and (*d*) same field as above, photographed after further processing for alkaline phosphatase (*d*, phase contrast microscopy). Myoid cells appear positive for both desmin and alkaline phosphatase. ×450.

tubulum and in the endothelium of interstitial blood capillaries and arterioles (Fig. 2). In the past, the peritubular localization of the reaction product has been interpreted as due either to the presence of the enzyme in myoid cells (Santiemma et al., 1978) or to the participation of extended strands of lymphatic endothelium to the constitution of the peritubular wall (Kormano, 1967; Dym and Fawcett, 1970). Very recently, while this work was in progress, Chapin et al. (1987) demonstrated for the first time that cultures of rat peritubular tissue contain cells that are specifically stained by alkaline phosphatase histochemistry. Moreover, alkaline phosphatase histochemistry has been used to screen for peritubular contaminants in Sertoli cell-enriched cultures (Anthony et al., 1987). Here we present evidence that the desmin-containing contractile "myoid" cells are responsible in vitro for alkaline phosphatase positivity in peritubular cell cultures (Fig. 5) and in cultures of tubular wall (Fig. 3). The cellular identification, hardly reliable in tissue sections due to resolution limitations, is readily obtained in vitro in monolayers in which double-labeling achieved by sequential immunostaining and processing for enzyme histochemistry can be observed in single cells. The observed co-localization of alkaline phosphatase and desmin positivity in the same cellular elements (Figs. 3, 5, 6) indicates alkaline phosphatase as a marker for myoid cells within cultures of rat peritubular and tubular tissue. Moreover, the distribution of the enzymatic reaction product over the entire extension of the cell membrane illustrates a particular morphology of the myoid cell in the first day after plating; on plastics, as well as on glass, myoid cells display a

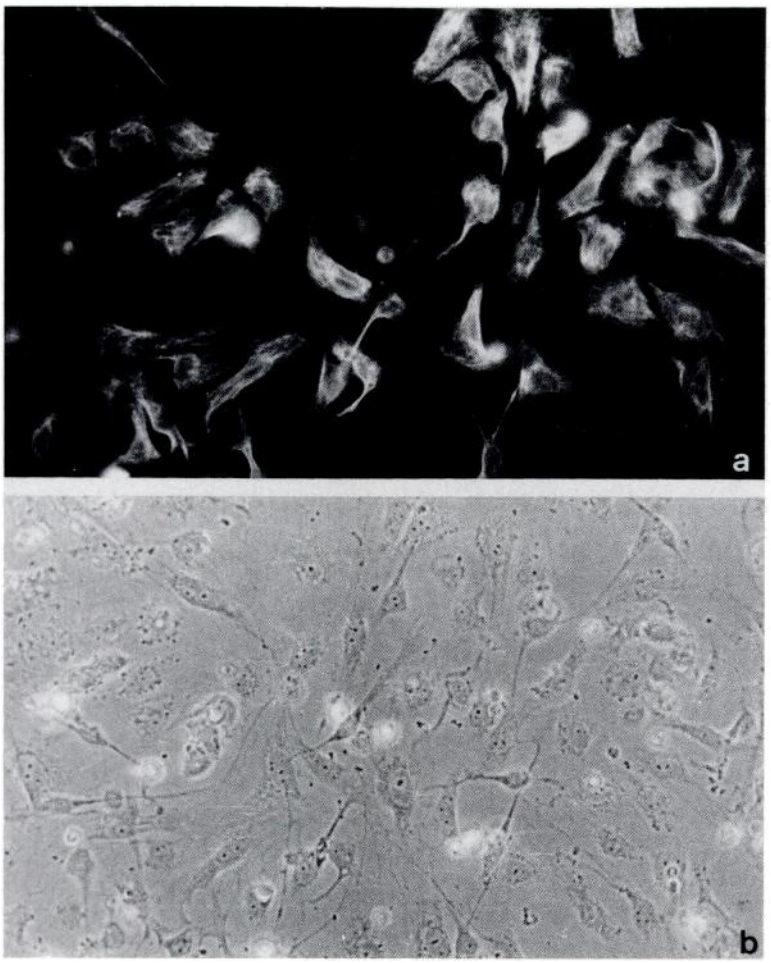

FIG. 4. Fluorescence (*a*) and phase-contrast (*b*) images of collagenase-dispersed peritubular cells cultured for 3 days and immunolabeled with anti-desmin antibody. A large part of the population appears negative to the marker. X1650.

characteristic polygonal morphology in early spreading. Such behavior suggests a possible delay in the onset of locomotion with respect to other cells, most of which assume an oriented shape very rapidly (Figs. 5 and 6). This is, to our knowledge, the first report of a morphological heterogeneity in peritubular cell cultures, which is by this method easily detected with the light microscope and which allows for rapid screening of myoid cells. Our results, both with desmin and with alkaline phosphatase positivity,

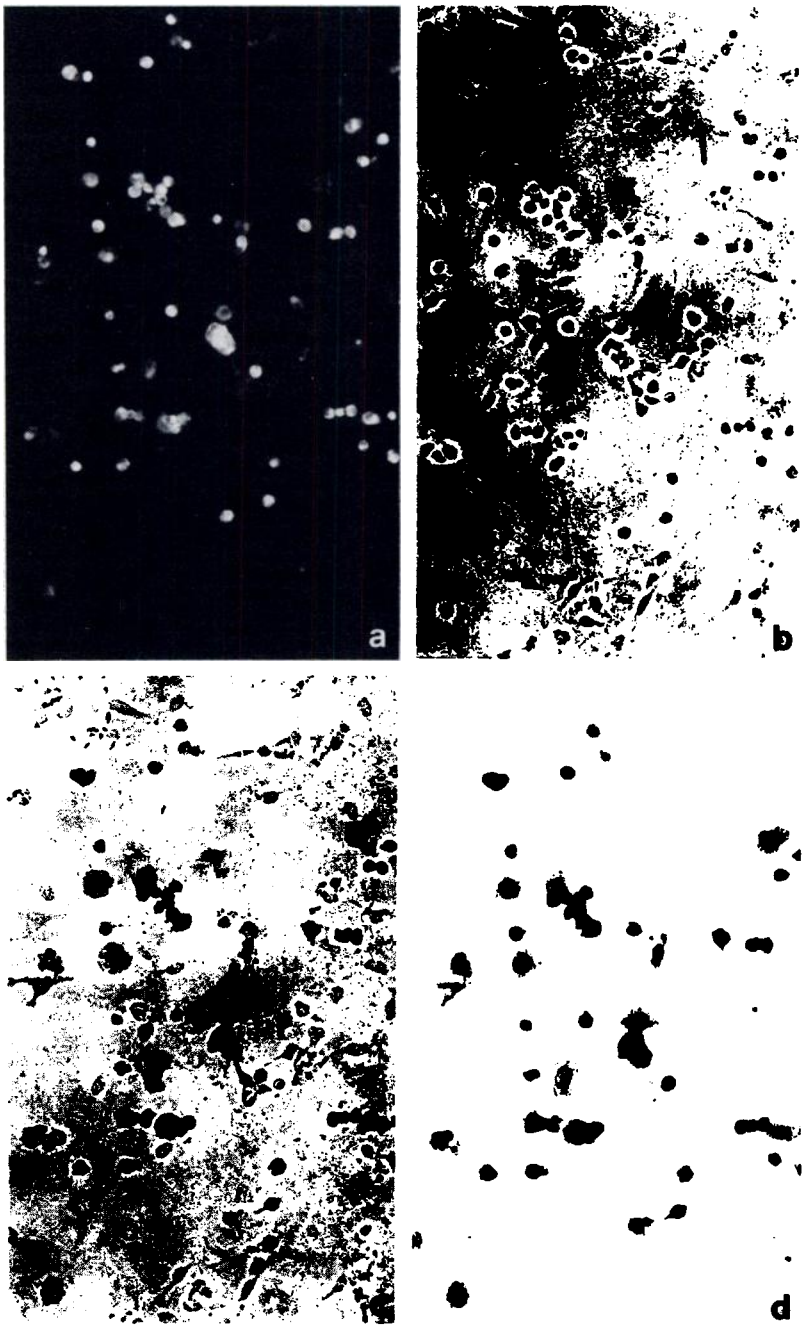

FIG. 5. Peritubular cell suspension after culturing for 24 h on glass chamber slide. (*a*) and (*b*) Photographed after immunostaining for desmin; (*c*) and (*d*) photographed after subsequent processing for alkaline phosphatase. (*b*) and (*c*), Phase-contrast optics. Only desmin-positive cells are positive for alkaline phosphatase (*arrows* point to non-myoid, negative cells). Although spreading on glass is retarded for most myoid cells, a number of them, after processing for the surface enzyme activity, display a wide phase-lucent halo, apparently devoid of desmin filaments. ×550.

provide evidence of a significant variability in percentage of myoid cells. Moreover, we find this cell type to represent only 30% to 50% of the cell population in vitro. These are relevant points, since data on peritubular cells in vitro and on their interaction with Sertoli cells have originated from different laboratories that often used quite different isolation and culturing protocols (Tung and Fritz, 1977; Cameron and Markwald, 1981; Kierszenbaum et al., 1986). Moreover, since the duplication rate in vitro of the single cell types present in culture is not known, we cannot exclude that the actual ratio of myoid vs. non-myoid cells may vary significantly with experimental time and after subculturing. In recent years, evidence has accumulated rapidly (Skinner and Fritz, 1985; Cameron and Snydle, 1985; Fritz and Tung, 1986; Hadley et al., 1985) in favor of a physiological role of peritubular cell products on seminiferous epithelium functions, a matter that, in our opinion, emphasizes the need for further cell characterization. Alkaline phosphatase histochemistry can contribute to the screening of peritubular cell preparations in different

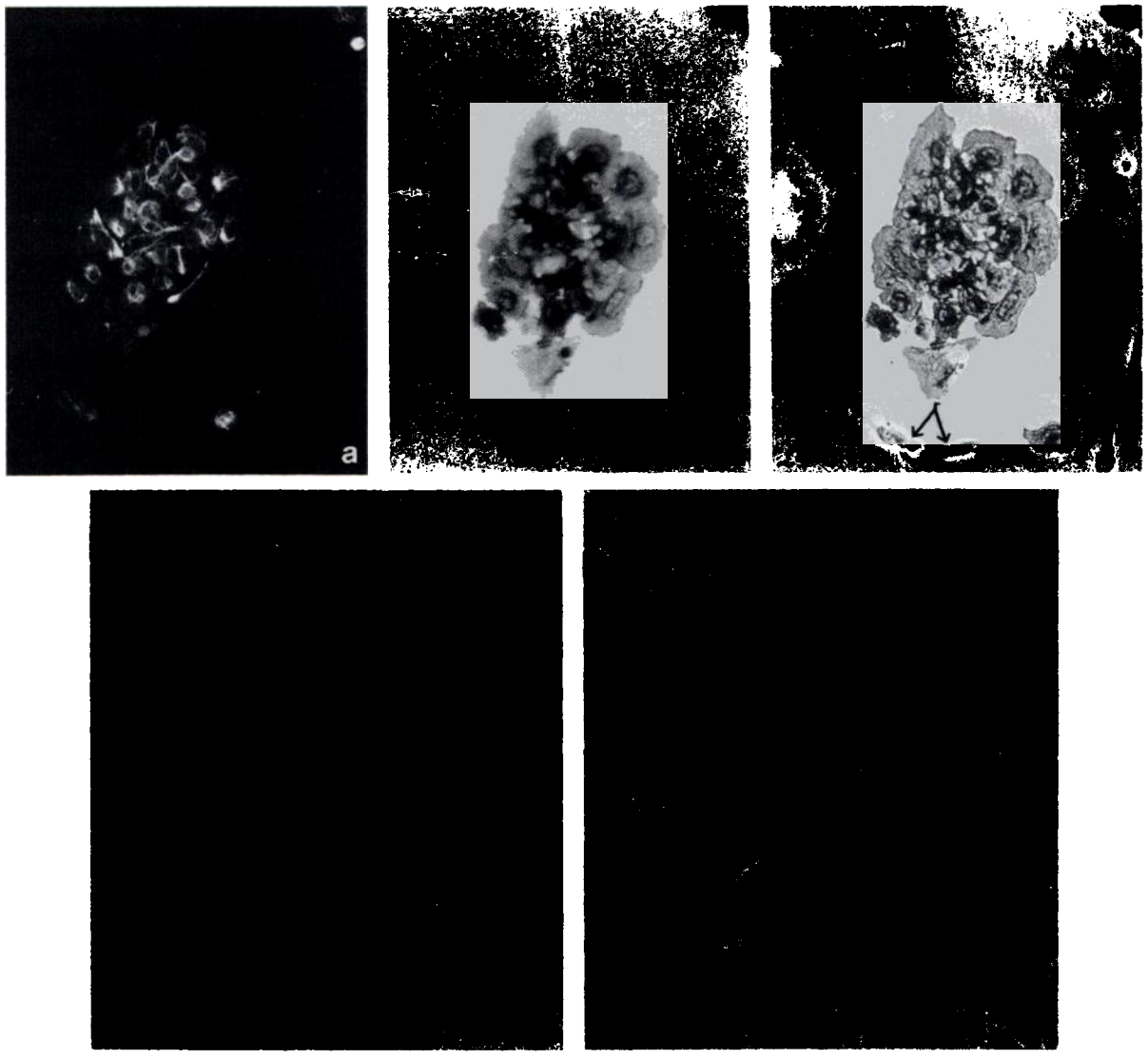

FIG. 6. Peritubular cell suspension after 24 h culture on a plastic substrate that allows more rapid spreading of myoid cells than glass. (*a*) Photographed after immunostaining for desmin; (*b*) and (*c*) same cells after subsequent processing for alkaline phosphatase activity. In (*c*) (phase-contrast optics) note elongated cells negative for both markers (*arrows*). The different morphology of alkaline phosphatase-positive vs. -negative cells in the first culture day is apparent in (*d*) and (*e*) (*e*, phase-contrast optics). *a, b, c,* ×750; *d, e,* ×650.

experimental conditions, to their standardization, and perhaps can foster their separation into purified cell classes.

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