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Leydig Cells Do Not Have Fc Receptors

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It has been reported that Leydig cells have Fc receptors, which traditionally have been considered markers specific for macrophages and polymorphonuclear leukocytes. The purpose of this investigation was to study further this phenomenon and also to determine if Leydig cells and macrophages could be separated from each other either by density gradient centrifugation using Percoll or by differential detachment with trypsin treatment of cultures of crude interstitial preparations. Interstitial cells were obtained by collagenase digestion of rat testis and established in culture. These cultures were reacted for 3β-steroid dehydrogenase and Fc receptor and viewed with phase contrast microscopy. No individual cells were positive for both steroid dehydrogenase activity and Fc receptors. The order in which the cells were stained for these two markers did not influence the results. Trypsin treatment of these crude interstitial cultures removed over 90% of the Leydig cells and approximately 20% of the macrophages. Macrophages were located in the same portion of Percoll gradients as the less dense (Population I) Leydig cells, while Leydig cells found in the dense area of the gradient (Population II) were not contaminated with macrophages. These studies indicate that Leydig cells do not have Fc receptors and that a subpopulation of Leydig cells can be isolated free of macrophages using density gradient centrifugation.

Key words: Leydig cells, macrophages, Fc receptors, steroid dehydrogenase.

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We have recently demonstrated that cultured Leydig cells secrete more testosterone (T) when treated with medium "conditioned" by testicular macrophages than with control medium (Yee and Hutson, 1985c). The testicular macrophages used in that study were prepared by collagenase digestion and differential attachment techniques. Macrophages were identified by demonstration of surface receptors for the Fc portion of immunoglobulin, which is a marker commonly used to identify such cells in other tissues (Diesselhoff-den Dulk and van Furth, 1981). Although the most common marker for Leydig cells is 3β-steroid dehydrogenase activity, it has also been reported that Leydig cells have Fc receptors (Molenaar et al, 1984; Wolpe and Mather, 1984). To interpret properly *in vitro* studies on the interaction between Leydig cells and testicular macrophages, specific and valid markers for each of these two testicular cell types must be established. Therefore, the first aim of the present investigation was to determine if individual cells from the interstitial compartment of the testis have both steroid dehydrogenase activity and Fc receptors. These studies examined the validity of using Fc receptor and steroid dehydrogen-

ase activity as markers for macrophages and Leydig cells, respectively. The second goal was to compare the location of Fc receptor-positive cells to the location of steroid dehydrogenase-positive cells obtained from Percoll gradients. Results from these studies provide useful data for those studying subpopulations of Leydig cells, since macrophages may be a contaminant of a specific subgroup. Finally, studies were conducted to determine if macrophages can be removed from culture substrates with trypsin treatment. Although it is commonly thought that macrophages cannot be removed by such treatment, this could be a potentially useful technique, simpler than density gradient centrifugation, to prepare Leydig cell cultures free of macrophages.

Methods

Preparation of Cells From the Interstitial Tissue

Two adult Wistar rats per experiment were killed by cervical dislocation and each testis was removed and gently decapsulated (without cutting tubules) in Dulbecco's phosphate-buffered saline (K.C. Biological, Lenexa, KS; PBS, pH 7.2) containing 1 mM calcium, 0.1 mM $MgCl_2$ and 0.1% bovine serum albumin (PBS/BSA). The testicular tissue was then transferred to a 125-ml Erlenmeyer flask containing 50 ml PBS/BSA plus 100 units/ml collagenase (Sigma Chemical Co., St. Louis, MO, Type I) and incubated in a shaking water bath at 34 C at 120 cycles/min for 10 minutes. The tubules were then allowed to settle for 2 min and the supernatant was centrifuged for 5 min at 1000 × g. The supernatant was discarded and the pellet resuspended in Dulbecco's modified Eagle's medium (Sigma Chemical Co., 1 g/l glucose) with 10% heat-inactivated fetal calf serum and 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone (Sigma). All the above procedures were conducted using sterile techniques.

Validation of Specific Cell Markers

Cells from the collagenase digestion were plated in 60-mm plastic Petri dishes (Nunc, Vangard Int, Neptune, NJ) containing 4 ml of complete medium and cultured for 24 hours at 33 C in a water-saturated atmosphere of 95% air and 5% CO_2. The cells were then washed once with PBS/BSA and reacted for steroid dehydrogenase activity and the presence of Fc receptors as described below. Positive cells were counted using a gridded eyepiece calibrated to a stage micrometer. Ten randomly selected 62,500-μm^2 areas were counted/dish. In some experiments the cells were first assayed for steroid dehydrogenase followed by the Fc receptors assay, while in other experiments the cells were first assayed for Fc receptor followed by the steroid dehydrogenase assay to determine if the order in which the cells were assayed for a specific marker influenced the results. This experiment was conducted 3 times.

Location of Macrophages on Percoll Gradients

Cells from the collagenase digestion as described above were layered onto a solution of 55% Percoll (Sigma Chemical Co.) and 45% PBS/BSA (30 ml total volume) and centrifuged at 30,000 × g for 15 min as described by Georgiou and Payne (1987). Approximately 1-ml fractions were pumped from the bottom of the gradients (fraction #1 representing the bottom) and washed free of Percoll by dilution with PBS/BSA and centrifugation. The fractions were split into two equal portions and plated into 35 × 10-mm plastic Petri dishes (Corning Glassworks, Corning, NY) in 1 ml complete medium. The cells were cultured for 24 hours and reacted for 3β-steroid dehydrogenase or Fc receptor as described below. In some experiments, the cells were plated into 60-mm Nunc Petri dishes in 4 ml complete medium and each dish stained for both steroid dehydrogenase and Fc receptor. Positive cells were counted using a gridded eyepiece calibrated to a stage micrometer. The medium was assayed for T by radioimmunoassay (Radioassay Laboratories). This experiment was conducted 3 times.

Trypsin Treatment

Cells obtained from the collagenase digestion were plated into 60-mm diameter Nunc plastic Petri dishes and cultured 24 hours in complete culture medium. The cells were then rinsed 2 × with PBS/BSA and then digested with 0.25% trypsin in PBS for 15 minutes at 33 C to determine if trypsin treatment preferentially would remove Leydig cells. The trypsin solution was then removed and centrifuged at 700 × g for 10 minutes. The pellet was resuspended in complete medium and plated into 35-mm Petri dishes. Complete medium was added to the cells still attached after trypsin treatment. Both sets of cells as well as an original dish that had not been treated with trypsin were cultured for 2 additional hours and then reacted for 3β-steroid dehydrogenase activity and the presence of Fc receptors as described below. Positive cells were counted as described above. This experiment was conducted once.

Fc Receptor Assay

Bovine red blood cells were coated with immunoglobulin specific for such cells by incubating (per dish) 100 μl of a 10% suspension of washed red blood cells (Cooper Biomedical, Malvern, PA) with 2 μl anti-bovine red blood cells (Cooper Biomedical, 21.6 mg/ml) in 1 ml PBS/BSA at 37 C for 5 minutes. The coated cells were then diluted with 10 volumes of PBS/BSA and centrifuged at 1000 × g for 5 minutes. The cells were then resuspended in 1 ml PBS/BSA, added to cultured cells and incubated for 10 minutes at 33 C. The cells were then washed with PBS/BSA to remove unattached red blood cells. Controls included uncoated red blood cells and coated red blood cells incubated with cultured cells in the presence of excess anti-RBC (100 μl/ml).

3β-Steroid Dehydrogenase Activity

Cells were incubated for 1 hour with 0.7 M phosphate buffer (pH 7.4) containing 1 mg/ml nicotinamide, 6 mg/ml

TABLE 1. Percent of Total Cells Represented by Leydig Cells and Macrophages*

Treatment	Fc Receptor	SDH	Other Cells
Untreated cultures	21.2	8.5	70.2
Cell remaining after trypsin	17.2	0.7	82.1
Cells removed by trypsin	6.3	17.1	76.6

*Cells from crude collagenase digestion were plated into 60-mm Petri dishes and cultured overnight. The cells were then either left untreated or digested with 100 units/ml collagenase in PBS/BSA for 15 minutes at 33 C. The cells in the trypsin solution were then washed and reestablished in culture for 2 hr. The cells remaining attached were also washed and cultured an additional 2 hr. All three groups were then reacted for Fc receptor and steroid dehydrogenase. The number of positive cells/dish was determined and expressed as a percent of the total cells present. SDH = steroid dehydrogenase-positive cells. Total number of cells in untreated cultures was 1.65×10^6.

nicotinamide adenosine dinucleotide, 100 μg/ml dehydroepiandrosterone (10 μl of a stock of 10 mg/ml ethanol), and 1.5 mg/ml nitro blue tetrazolium. The cells were then rinsed with PBS/BSA. Controls included omission of dehydroepiandrosterone and/or fixing cells with 1% formalin for 2 hours prior to staining.

Results

Identity of Macrophages and Leydig Cells

Cultures of crude interstitial cells obtained by collagenase digestion were approximately 21.3% positive for Fc receptor and 8.5% positive for steroid dehydrogenase activity (Table 1). No cells were positive for both Fc receptors and steroid dehydrogenase activity (Fig. 1 and Fig. 2). The order in which the cells were stained for steroid dehydrogenase or Fc receptor had no influence on the results.

Location of Macrophages and Leydig Cells on Percoll Gradients

The majority of the cells were located on the top of the gradient in fractions 28 to 32 (Fig. 3). Two populations of steroid dehydrogenase-reactive cells were observed; one in fractions #1 to 6 (density of 1.09 to 1.12 g/ml) and the other in fractions #26 to 32 (density of 1.05 to 1.065 (Fig. 4). The macrophages were found only in the less dense area of the gradients in fractions 29 to 32 (density of 1.05 to 1.06, Fig. 4). The concentration of T in the medium was well correlated with the number of steroid dehydrogenase-positive cells (Fig. 5).

Specific Removal of Cells With Trypsin

Trypsin treatment for 15 minutes of crude interstitial preparations cultured for 24 hours removed 92% of the Leydig cells. Twenty percent of the macrophages were removed by this treatment (Table 1).

Discussion

Macrophages have been demonstrated by morphologic techniques to be a normal cell type within the interstitial compartment of the testis (Clegg and MacMillan, 1965; Kormano, 1968; Christensen and Gillim, 1969; Connell and Christensen, 1975; Clark, 1976; Sinha et al, 1977; Wing and Lin, 1977; Ohata, 1979), representing approximately 20 to 25% of the cells (Miller, 1982; Niemi et al, 1986). They can be isolated from the testis and exhibit a number of characteristics common to macrophages in other tissues, including: 1) the presence of Fc and Ia receptors, 2) the ability to phagocytize and kill pathogenic bacteria, 3) nonspecific esterase activity, 4) the ability to attach rapidly to culture substrates, and 5) typical morphologic features (Miller, 1982; Yee and Hutson, 1983, 1985a, 1985b, 1985c; Miller et al, 1983, 1984; Niemi et al, 1986). However, two reports suggest that Leydig cells share with the macrophage the ability to bind the Fc portion of immunoglobulin. Molenaar et al (1984) reported that Leydig cells from adult rats possessed Fc receptors while Leydig cells from immature rats were negative for this receptor. They suggested that the functions of Leydig cells and macrophages change during maturation with some functions being in common. Results from the present investigation strongly indicate that Leydig cells from adult rats do not have Fc receptors. These results support the similar conclusion by Niemi et al (1986), who demonstrated that macrophages and Leydig cells could be specifically differentiated *in situ* using double staining for a macrophage antigen and steroid dehydrogenase. Our results extend these observations indicating that Leydig cells and macrophages retain this degree of specificity when established in culture. We are unable to speculate why our results differ from those of Molenaar et al (1984). In the report by Wolpe and Mather (1984), interstitial cell preparations from immature mice were trypsinized and the cells thus removed from culture substrates were analyzed for hCG binding and the presence of Ia and Fc receptors. It was reported that the cells removed by trypsin were positive for steroid dehydrogenase activity and Fc receptors but not for Ia antigen. However, it is not known if these two markers were found in the same cell type. It was assumed in that report that macrophages cannot be

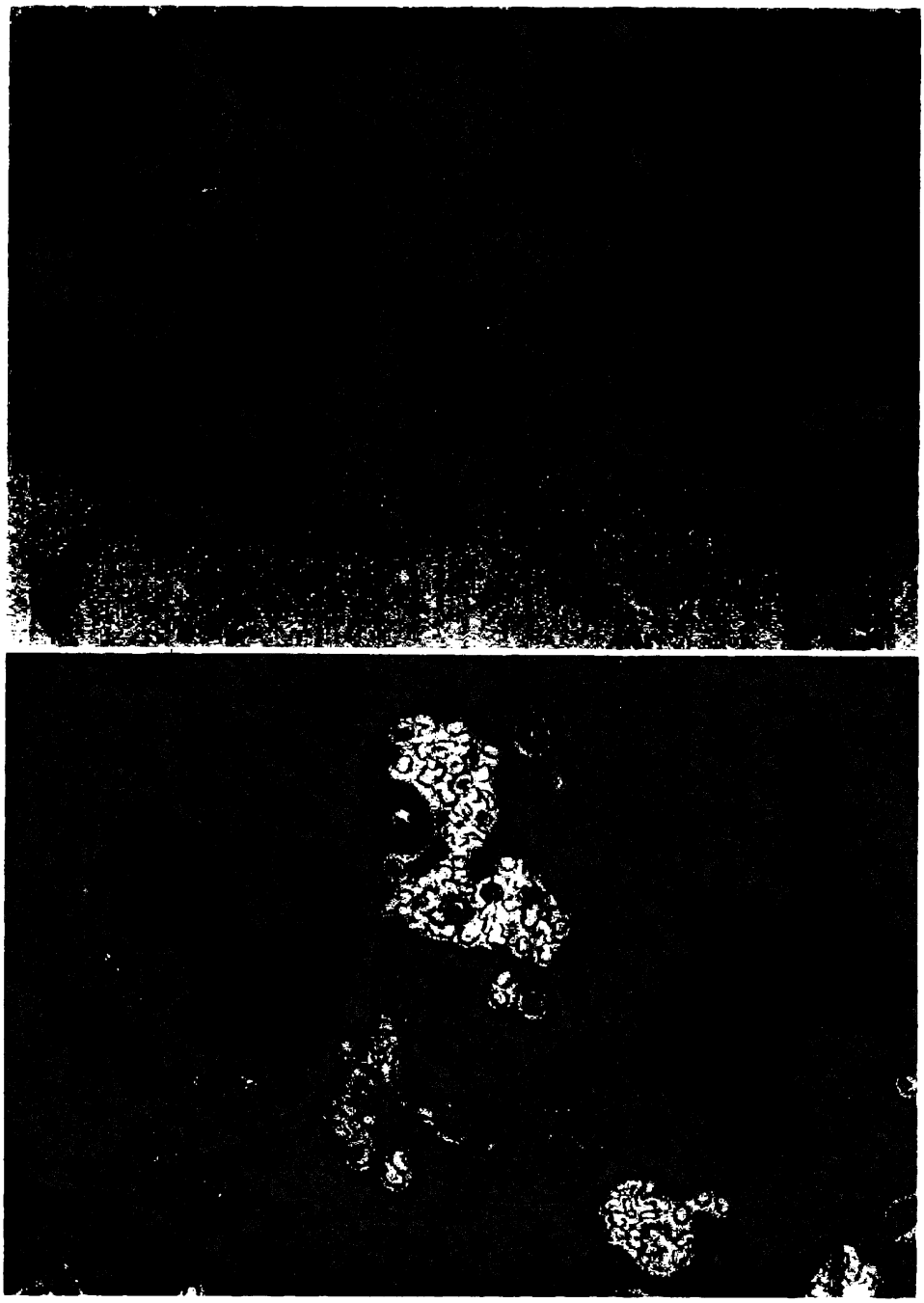

Fig. 1. Cells from the crude collagenase digestion were washed with complete medium and plated into 100-mm diameter Petri dishes and cultured for 24 hours. The cells were then reacted for steroid dehydrogenase and Fc receptor. No individual cells contained reaction product for both steroid dehydrogenase and Fc receptors. The bottom panel was photographed with phase contrast optics while the top panel was photographed with bright-field optics. Cells positive for steroid dehydrogenase contain dense reaction product in their cytoplasm (arrows). Cells with Fc receptors bound IgG-coated red blood cells (arrow heads) × 400.

removed by trypsin treatment and therefore the data were interpreted to indicate that Leydig cells have Fc receptors. Our results clearly demonstrate that rat testicular macrophages (cells positive for Fc receptor but not steroid dehydrogenase activity) can be removed by trypsin treatment and can be reestablished in culture. Therefore, macrophages may have contaminated the presumptive Leydig cell preparations, as described by Wolpe and Mather (1984). However, a major difference between our studies and that of Wolpe and Mather (1984) is that we have used cells from the rat while theirs was conducted on immature mice. Nonetheless, it is clear that trypsin treatment of crude interstitial cell preparations is not a suitable method for purifying Leydig cells. Not only are macrophages removed by this treatment but the possibility of enzyme damage to surface receptors must be considered.

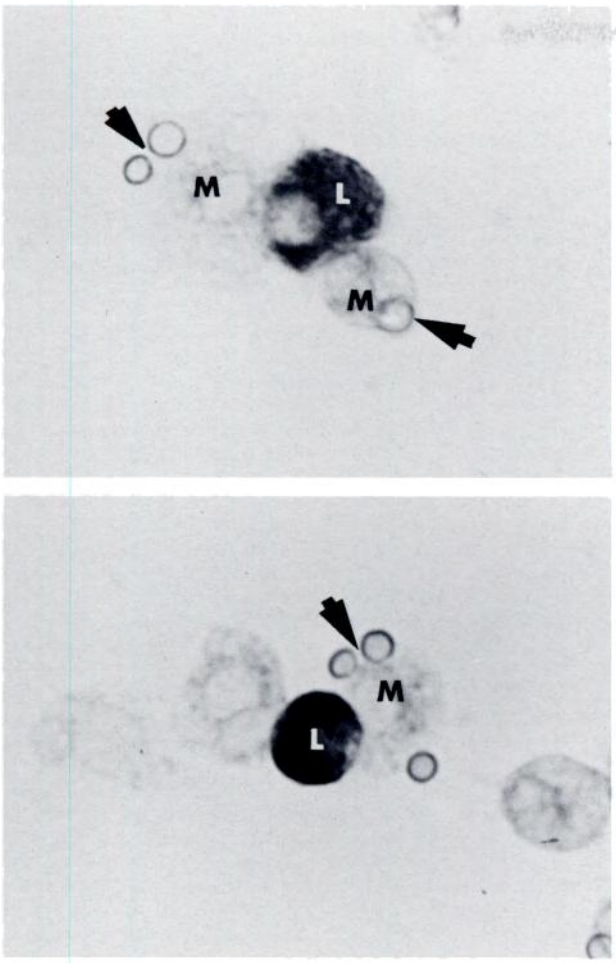

Fig. 2. Cells from the "light" area on the gradients (fractions 30 and 31) were collected and established in a single 60-mm culture dish. The cells were then stained for steroid dehydrogenase and Fc receptors. It can be seen in these bright field micrographs that individual cells from this area of the gradient are either positive for steroid dehydrogenase or Fc receptors while none are positive for both markers. Arrows indicate red blood cells. L = Leydig cell. × 800.

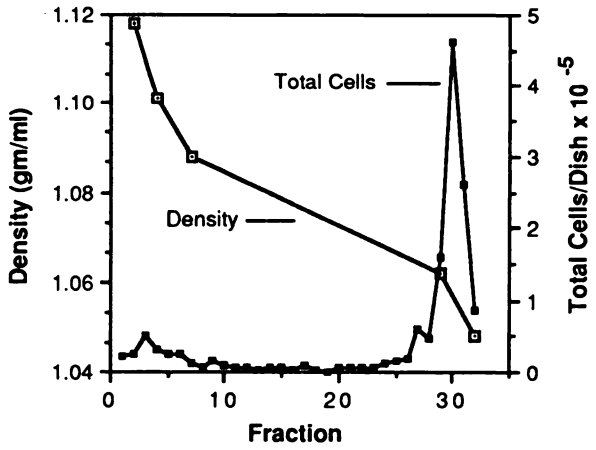

Fig. 3. This figure illustrates the location of all cells capable of attaching to the culture substrate for each fraction. Cells were grown for 24 hours and then counted using a gridded eyepiece calibated to a stage micrometer. The density of the gradient was estimated using density marker beads (Sigma) in a separate gradient prepared and centrifuged simultaneously with the gradient used to prepare the cells. Fraction 1 represents the bottom of the gradient and was the first fraction to be pumped from the tube.

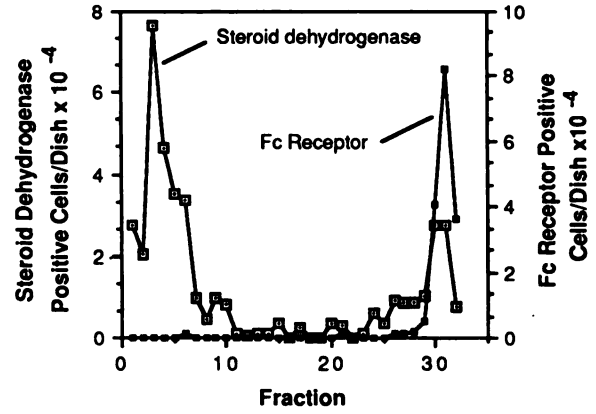

Fig. 4. This figure illustrates the location of steroid dehydrogenase-(SDH) positive cells (Leydig cells) in relation to the Fc receptor-positive cells (macrophages). Individual fractions from the gradient were split into two equal portions. Each portion was plated into a 35-mm diameter Petri dish and cultured for 24 hours. One set of culture dishes was stained for steroid dehydrogenase and the other reacted for Fc receptor. The most dense Leydig cells were virtually free of macrophages while the lightest Leydig cells were located in the same fractions as the macrophages.

Leydig cell populations have been studied for several years using density gradient centrifugation (Janszen et al, 1975; Payne et al, 1980; Browning et al, 1981; Dehejia et al, 1982; Cooke et al, 1983; Aquilano and Dufau, 1984; Hedger and Eddy, 1986; Bhalla et al, 1987a, 1987b). However, the precise location of macrophages, determined by Fc receptor binding rather than equivocal morphologic methods, on these gradients in relation to Leydig cells has not been studied. The present studies indicate that macrophages are located in the same area as Population I Leydig cells as described by Georgiou and Payne (1987). Our results indicate that the Leydig cells corresponding to Population II, which are found in the most dense portion of the gradients, are virtually free of macrophages. Therefore, Population II Leydig cells may represent the most suitable subgroup for studying interactions between macrophages and Leydig cells *in vitro*. This

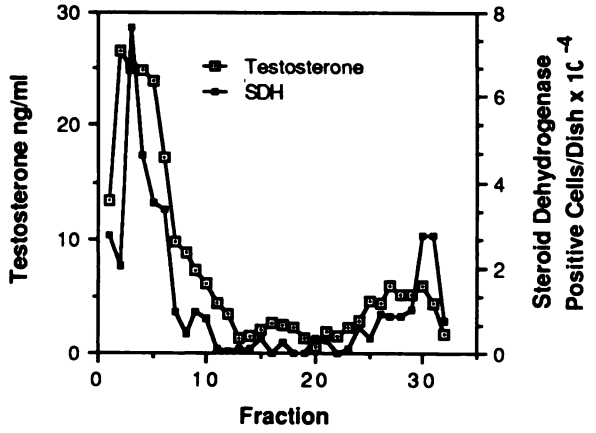

Fig. 5. This figure illustrates the location of steroid dehydrogenase-positive cells on Percoll gradients and their ability to produce testosterone. Cells were grown 24 hours prior to staining and radioimmunoassay for T. The slight increase in efficiency of T production for cells of fractions 30 to 31 was not a reproducible phenomenon in two separate experiments.

population is the same as that described by Browning et al (1981), which was used in our previous work establishing a functional relationship between Leydig cells and macrophages (Yee and Hutson, 1985c). The presence of macrophages in Population I Leydig cells may complicate interpretations of studies involving the comparison of this subgroup to Population II cells (that are free of macrophages). It cannot be assumed that differences in populations of Leydig cells are intrinsic without determining if the presence of macrophages is involved. This further complicates attempts to validate the authenticity of various populations of Leydig cells, since some authors attribute the presence of the "lighter" (Population I) Leydig cells to damage occurring during isolation procedures (Dehejia et al, 1982; Cooke et al, 1983; and Aquilano and Dufau, 1984). However, others indicate that two populations of Leydig cells exist that are unrelated to isolation artifacts (Georgiou and Payne, 1987; Bhalla et al, 1987a, 1987b).

It is concluded that Leydig cells and macrophages can be easily distinguished from each other using histochemical and immunologic methods specific for Fc receptor and 3β-steroid dehydrogenase activity. A population of Leydig cells with a density of approximately 1.09 to 1.12 can be separated free from macrophages using density gradient centrifugation, while macrophages can be easily separated from nearly all Leydig cells, regardless of density, by differential attachment techniques (Yee and Hutson, 1985a, 1985b).

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3rd International Conference on the Control of the Onset of Puberty
Amsterdam, May 7–10, 1989

The 3rd International Conference on the Control of the Onset of Puberty will be held in Amsterdam on May 7–10, 1989. In addition to the traditional comparative and clinical approaches to the study of puberty, sessions will be devoted to an examination of the neuroendocrine basis of the hypothalamic restraints underlying other physiologic states of diminished pituitary gonadotropin secretion. Also, an entire session will be devoted to the question of how recent advances in the neurosciences and molecular endocrinology might be applied to the problem of puberty. A discussion of the application of mathematical modeling and a session for poster presentations are also planned.

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The IVth International Congress of Andrology
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The IV International, to be held in Florence, Italy from May 14 to 18, 1989, Congress is organized in Plenary Lectures, Symposia, and mini-posters presentation.

The topics of the meeting will be 1) Gene expression and growth factors in the testis; 2) Local control mechanisms in the testis 1; 3) Neuroendocrine factors in testicular control; 4) Pituitary–testicular interactions; 5) Sperm maturation and sperm function; 6) New technologies in reproductive medicine; 7) Antifertility methods for men; 8) Prostate; 9) Impotence; 10) Diagnostic approaches to male infertility; 11) Management of male infertility; 12) Testicular tumours; 13) Sexual differentiation.

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