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THE IMMATURE RABBIT TESTIS : PRESENCE OF TWO DISTINCT POPULATIONS OF LEYDIG CELLS

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SUMMARY : In the immature rabbit testis (78 days), after a collagenase treatment (0.05 %), 20 min at 32°C in Ham F12/DME medium and centrifugation of the crude testicular cell preparation obtained on a discontinuous Percoll gradient, we demonstrated the presence of two enriched Leydig cell populations (CLI : 47 % and CLII : 76 %) which viability is higher than 95 %. In basal conditions (Ham F12/DME medium, 5h at 32°C), Leydig cells of CLI synthesize 3 fold more testosterone than Leydig cells of CLII (32 ± 4 and 9.5 ± 0.1 ng/10^6 Leydig cells) ; in addition the cells of CLI are twice as much sensitive to hCG as these of CLII. After a preincubation of 20h followed by an additional incubation of 5h, a 3 fold increase of the testosterone outputs were recorded in both Leydig cell fractions. Taking into account the % of 3β-HSD positive cells in each population, it is obvious that the Leydig cells of CLI are very potent steroidogenic secreting cells.

RÉSUMÉ : Présence de deux populations distinctes de cellules de Leydig dans le testicule de lapin immature. *Dans le testicule de lapin immature (78 jours), après un traitement enzymatique à la collagénase (0.05 %) pendant 20 min à 32°C et centrifugation des cellules testiculaires obtenues sur gradient de Percoll, nous démontrons l'existence de deux populations cellulaires distinctes enrichies en cellules de Leydig (CLI : 47 % et CLII : 76 %) dont la viabilité est supérieure à 95 %. Les cellules de Leydig de CLI sythétisent 3 fois plus de testostérone que celles de CLII (32 ± 4 et 9,5 ± 0,1 ng/10^6 cellules de Leydig) quand elles sont incubées 5h à 32°C dans du milieu Ham F12/DME ; les cellules de CLI sont en outre, 2 fois plus sensibles à l'hCG que celles de CLII. Après une préincubation de 20h suivie par une incubation de 5h, les productions de testostérone sont augmentées de 3 fois en moyenne et les cellules de CLI restent les plus efficaces, surtout si l'on tient compte du pourcentage de cellules 3β-HSD positives par rapport à celui de CLII.*

INTRODUCTION

It is well established that the regulation of steroidogenesis in mammalian Leydig cells involves the interaction of LH/hCG with its membrane receptor which induces an activation of the adenylate cyclase and finally an increase of steroid synthesis. Indeed, together with FSH, testosterone is required for the complete development and the maintenance of spermatogenesis (STEINBERGER, 1971). In order to better understand the hormonal control of the Leydig cell testosterone production, established methods using the density gradient centrifugation technique with either metrizamide or Percoll for isolating enriched fractions of testicular cells from rat (LAWS *et al.*, 1985 ; PAPADOPOULOS *et al.*, 1985 ; GEORGIOU and PAYNE, 1987) and mouse (COOKE *et al.*, 1981) have gained considerable importance in the past decade. Two populations of Leydig cells have been characterized in rat (BHALLA *et al.*, 1987), equine (ALMAHBOBI *et al.*, 1988) and human (PAPADOPOULOS *et al.*, 1987 ; LEJEUNE *et al.*, 1993 ; MAILLARD *et al.*, 1994) testes and numerous reports have bring insights on the effects of seminiferous secreted factors in addition to LH, on the regulation of Leydig cell function (see for review CARREAU, 1993). We reported herein the presence of two Leydig cell populations in the immature rabbit testis and the effect of hCG on the production of testosterone by these cells.

MATERIALS AND METHODS

Preparation of Leydig cells

Immature rabbit (mean age : 78 ± 1.0 days, n = 54) were from a French commercial breed Hyla. The testes were collected at the slaughterhouse, cleared of fat, then the albuginea was removed and the tissues were coarsely minced before being washed several times with an Ham's F12-DME medium (1:1, v/v) containing penicillin (0.12 g/l) and streptomycin (0.20 g/l). After an enzymatic treatment with

collagenase-dispase (0.05 %), soybean trypsin inhibitor (0.005 %) and deoxyribonuclease (0.001 %) for 20 min at 32°C in Ham's F12-DME, followed by several washes and settlings, the crude testicular cell suspension was filtered through nylon gauze (30 Mesh). This heterogeneous cell preparation was then layered on the top of discontinuous Percoll gradient (20-80 %) and centrifuged (1100 g, 20 min, 18°C). The Leydig cells were characterized by a positive staining for the 3β-hydroxysteroid dehydrogenase activity (3β-HSD+) and their ability to produce testosterone in the presence of hCG as reported elsewhere (PAPADOPOULOS *et al.*, 1985). The testicular cell viability was appreciated using the Trypan blue exclusion test.

Leydig cell incubations and testosterone measurements

The cells from each fraction collected after centrifugation on the Percoll gradient, were incubated in Ham's F12-DME either separately or per groups of cell fractions (CLI : fractions 4 to 6 ; CLII : fractions 8 to 10) during 5h or 20 + 5h (preincubation of 20h) with or without increasing concentrations (0.1 to 10 IU/ml) of hCG at 32°C under air/CO_2 (95:5, v/v). The testosterone productions were determined by RIA in the cell culture media (PAPADOPOULOS *et al.*, 1985 ; ALMAHBOBI *et al.*, 1988) ; the intra and interassay coefficients of variation were 4 and 6 %, respectively and the sensitivity was 4 pg/tube.

Statistical analysis

All the data collected were expressed as means ± SEM. The statistical analyses were performed using the one-way analysis of variance (ANOVA) ; the subsequent differences between each treatment were estimated by the Student's t-test and considered as significant for $P<0.05$.

RESULTS AND DISCUSSION

In the rabbit testis, we demonstrated the presence of two enriched-populations of functional Leydig cells (CLI and CLII) isolated after Percoll gradient centrifugation, based on their capacity to produce testosterone under hCG stimulation (Fig. 1) and to show a positive staining for 3β-HSD (Fig. 2C). Moreover, we provided evidence that these two cell populations are not damaged since they kept a high viability (>95 %) after Percoll purification (Fig. 2B). The cells of fractions 1, 2 and 12 which are respectively represented by cell fragments and few Sertoli cells (fractions 1 and 2) and by red blood cells (fractions 12) are more damaged (45 and 85 % of dead cells, respectively).

Table 1 : Viability and 3β-hydroxysteroid dehydrogenase activity (3β-HSD) in the two Leydig cell populations (CLI and CLII) obtained from immature rabbit testis after Percoll gradient centrifugation.

	Fraction number	
	4 + 5 + 6 (CLI)	8 + 9 + 10 (CLII)
3β-HSD	46.80 ± 1.16	75.90 ± 1.21***
Viability	97.00 ± 1.36	94.70 ± 0.75

Results are given in percentage ; *** $P<0.001$ compared to CLI

Our results are in agreement with those reported in the rat by GEORGIOU and PAYNE (1987) and by BROWNE *et al.* (1990), in horse (ALMHABOBI *et al.*, 1988) and in human (SIMPSON *et al.*, 1987 ; LEJEUNE *et al.*, 1993 ; MAILLARD *et al.*, 1994) : there is likely two types of Leydig cells called "dark and light" in the testis of these mammalian species. The enriched population of Leydig cells (Table 1) localized in Percoll fractions 4 to 6 (CLI : mean density of 1.05 g/ml), contains highly sensitive Leydig cells which produce large amounts of testosterone (Fig. 1 and 3) after 5h of incubation under a saturating dose (5 IU) of hCG (150 ± 9 ng/10^6 Leydig cells). By contrast, the Leydig cells localized in fractions 8 to 10 (CLII : mean density of 1.07 g/ml) synthesize less testosterone (32 ± 0.1 ng) and are less sensitive to hCG (Fig. 1 and 3).

When preincubated 20 h (Fig. 4), the Leydig cells of each population produce 2-3 fold more testosterone in presence of hCG : the maximal output of testosterone (354 ± 9.5 ng, i.e. a 10 fold increase over the basal testosterone production) is obtained with Leydig cells of CLI incubated in presence of 0.1 IU of hCG. The Leydig cells of CLII incubated under the same conditions produce 4 fold less testosterone (86 ± 0.07 ng) in presence of a saturating dose of hCG (Fig. 4). Taking into account the % of 3β-HSD positive cells in each enriched population (46.8 *vs* 75.9 %, Table 1), the Leydig cells of CLI are more sensitive to hCG, and consequently, more potent steroidogenic secreting cells than Leydig cells of CLII. According to the results published by BHALLA *et al.* (1987 ; 1992), the two main populations of Leydig cells isolated from the rat testis are very different in terms of LH receptor mRNA, cyclic AMP and testosterone outputs ; however, it is the heavier Leydig

cells in the rat (fractions 8 to 10) which produce more testosterone (GEORGIOU and PAYNE, 1987) whereas it is the Leydig cells of fractions 4 to 6 which are more active in the rabbit as well as in human (MAILLARD *et al.*, 1994). This discrepancy is may related to the species studied and/or the calculation of the testosterone output which is based on the number of 3β-HSD positive cells as well as on the experimental conditions, namely cell purity, cell density and incubation conditions as suggested by ABAYASEKARA *et al.* (1991).

Whatever, as mentioned previously for horse Leydig cells (ALMAHBOBI *et al.*, 1988) and more recently for human Leydig cells populations (QURESHI and SHARPE, 1993), there is no correlation between the percentage of "dark and light" cells in testis and their capacities to produce testosterone.

It is of note that for a larger dose of hCG (10 IU), the Leydig cell testosterone production are decreased of 50 % in both populations. This phenomenon called "desensitization", is indeed well known for the steroidogenic cells and especially for the rodent Leydig cells (AQUILANO *et al.*, 1985) in which the cholesterol side chain cleavage (rate limiting step of the steroidogenesis) and/or the 17α-hydroxylase/17–20 desmolase enzyme activities (O'SHAUGHNESSY *et al.*, 1981) are decreased. In consequence, the Leydig cell testosterone production is reduced but this event may be overcome by the paracrine factors produced by the Sertoli cells of the seminiferous tubules (GACHIE and CARREAU, 1994).

In conclusion, we demonstrated the existence of two functional Leydig cell populations in the immature rabbit testis and the different steroidogenic potencies of these two types of Leydig cells represents a common feature in mammals such as the rat, mouse, horse and human. This testicular characteristics is likely related to different Leydig cells types or heterogeneity (COOKE *et al.*, 1981 ; LAWS *et al.*, 1985 ; BROWNE and BHALLA, 1991) in relation to the existence of a Leydig cell cycle via a seminiferous tubules control (BERGH, 1982) through the effect of some of the paracrine factors produced by the Sertoli cells under a germ cell control (BOUJRAD *et al., 1992* ; CARREAU, 1993 ; CARREAU *et al.*, 1994).

Figure 1 : Testosterone production (ng/10^6 Leydig cells) by the immature rabbit testicular cells obtained after centrifugation on Percoll gradient and incubated during 5h either in absence or in presence of 0.5 IU of hCG.

Results are means ± SEM (n = 3 experiments). CL I : Leydig cells I ; CL II : Leydig cells II ; * P<0.05 ; ** P<0.01 ; *** P<0.001 when compared to the respective basal value (control).

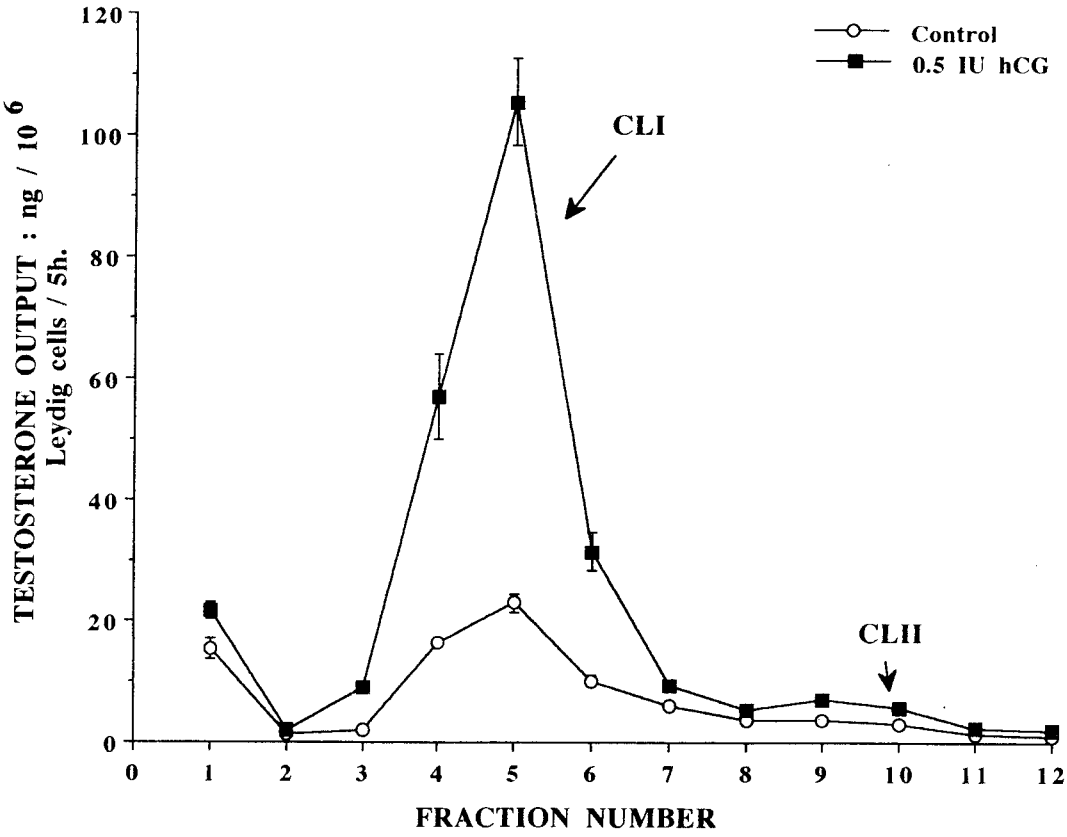


Figure 2 : Percentages of nucleated cells (A), of viability (B) and of 3β-hydroxysteroid dehydrogenase positive (C) cells (3β-HSD) in each fraction collected after centrifugation of immature rabbit testicular cells on Percoll gradient.

Columns having the same letter differ non-significantly, otherwise they differ from each other at $P<0.01$.

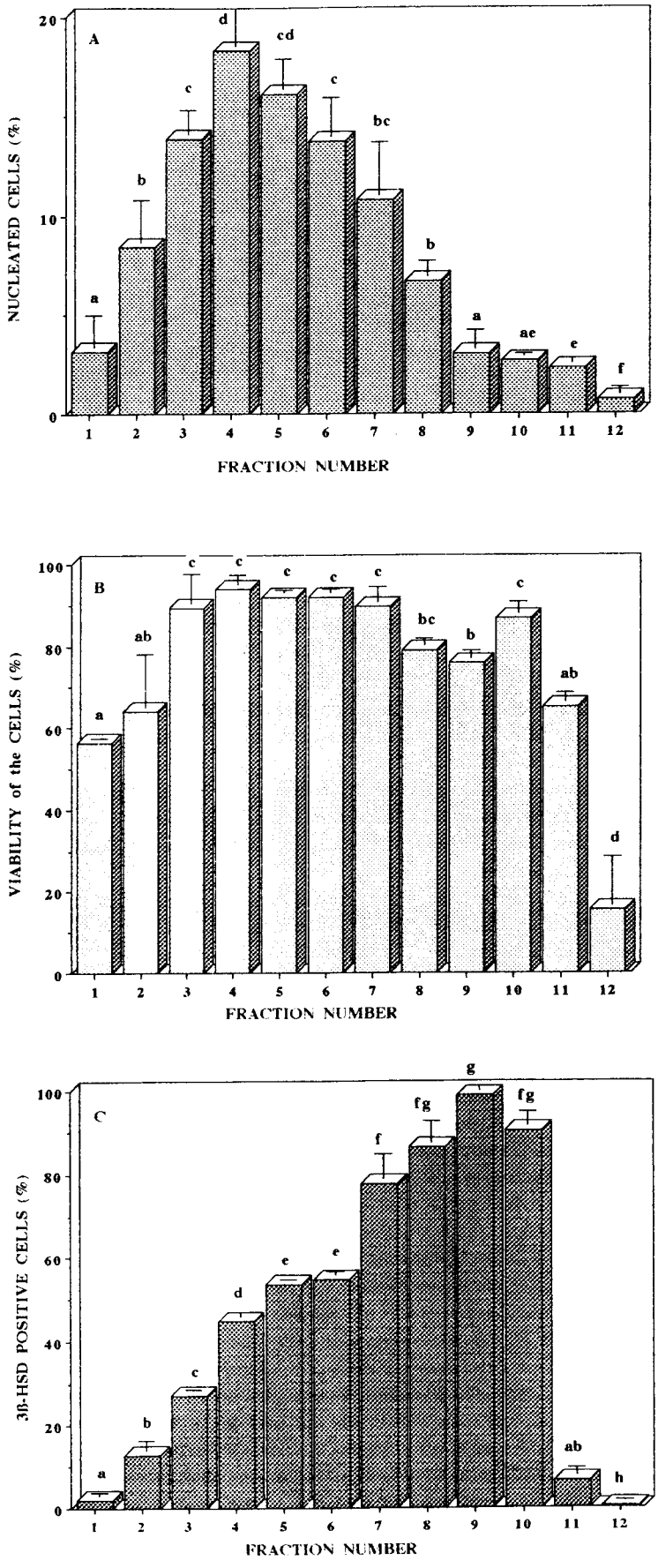


Figure 3 : Testosterone production (ng/10⁶ Leydig cells) in the two groups of rabbit Leydig cells (fractions 4-6 and fractions 8-10) incubated 5h with or without increasing amounts of hCG.
Results are means ± SEM (n = 5 experiments, each sample in duplicate). Columns having the same letter differ non significantly, otherwise they differ from each other at P<0.01).

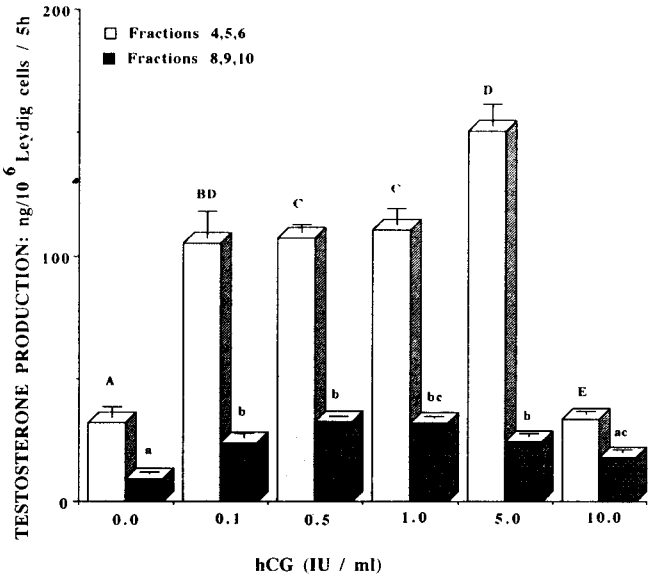
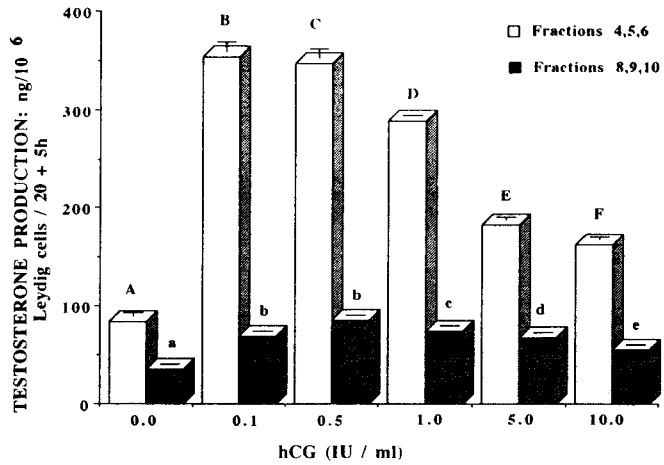


Figure 4 : Testosterone production (ng/10⁶ Leydig cells) in the two groups of rabbit Leydig cells (fractions 4-6 and fractions 8-10) preincubated 20h then for an additional period of 5h with or without increasing amounts of hCG.
Results are means ± SEM (n = 5 experiments, each sample in duplicate). Columns having the same letter differ non significantly, otherwise they differ from each other at P<0.01).



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