

Flow cytometric method to isolate round spermatids from mouse testis

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The purpose of this study was to isolate pure populations of round spermatids from mouse testis by flow cytometry followed by cell sorting. Cell suspensions from mouse testis were enriched in germ cells by centrifugation on a discontinuous Percoll gradient, then analysed using a FACScalibur flow cytometer measuring the cell size and density. A large and well-delimited population of cells (R1) expected to contain round spermatids was observed on the dot plot diagram. Sorted R1 cells were very homogeneous in size (~11 µm) and displayed the characteristic cytological aspect of round spermatids. Spermatid-specific gene expression was confirmed by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of R1 cells using primers for protamine 2 gene (*PRM2*) and *SP-10*. A positive signal for *SP-10* was obtained with a single cell using nested primers. The 5.5 kb transcript of *c-kit*, which is not expressed in spermatids, was not detected by nested RT–PCR, excluding a contamination with spermatogonia. Our results clearly established that flow cytometry followed by cell sorting allows the isolation of a highly homogeneous population of round spermatids from the testis.

Key words: flow cytometry/polymerase chain reaction/spermatids/spermatozoa

Introduction

Spermatogenesis is a complex process in which undifferentiated diploid stem cells develop into highly differentiated haploid cells, the spermatozoa. This process occurs in three main phases: spermatogonia proliferate by a series of mitotic divisions, spermatocytes undergo cytological differentiation during meiotic prophase and then proceed through reduction divisions to yield haploid spermatids (Bellvé and O'Brien, 1983); finally the spermatids pass through a complex phase (acrosome and tail formation, DNA packaging) which leads to the production of spermatozoa.

In-vitro embryo conception using spermatids has been performed in mammals (rodents: Ogura and Yanagimachi, 1993; Ogura *et al.*, 1993; Sasagawa and Yanagimachi, 1997; bovine: Goto *et al.*, 1996) and normal healthy offspring have been

reported (mouse: Ogura *et al.*, 1994, 1996; rabbit: Sofikitis *et al.*, 1994). Moreover, Kimura and Yanagimachi (1995a) have reported the development of normal mice from oocytes injected with secondary spermatocyte nuclei. Recently, human births following in-utero transfer of embryos obtained after intracytoplasmic injection of a human spermatid into a human oocyte have been reported (Fishel *et al.*, 1995; Tesarik *et al.*, 1995; Antinori *et al.*, 1997; Araki *et al.*, 1997; Vanderzwalmen *et al.*, 1997). Today, round spermatids may be proposed as substitutes for spermatozoa to achieve fertilization of human oocytes for the treatment of certain cases of azoospermia.

The analysis of spermatogenic cell populations is frequently complicated by the variety of somatic cells which are present in germ cell suspensions recovered after the dissection and the enzymatic digestion of seminiferous tubules. However, various procedures have been developed for isolating purified populations of germ cells from the testis in human and rodents: sedimentation at unit gravity (Bellvé *et al.*, 1977; Shepherd *et al.*, 1981; Wolgemuth *et al.*, 1985; Willison *et al.*, 1990), centrifugal elution (Meistrich, 1977; Bucci *et al.*, 1986; Blanchard *et al.*, 1991), separation using an immunoselection panning technique (Pelengaris and Moore, 1995), removal of contaminating somatic cells through adhesion to plastic dishes coated with lectin (Morena *et al.*, 1996) or flow cytometry followed by cell sorting (Mays-Hoopers *et al.*, 1995; Petit *et al.*, 1995; Suter *et al.*, 1997). However all these flow cytometry studies used fluorochromes to classify cells on the basis of their ploidy level, RNA content or mitochondrial changes. All these methods were able to select enriched round spermatid populations. Staining procedures using immunoglobulins, conjugated lectins or DNA intercalants to identify spermatids must be refuted if they have to be microinjected into oocytes after cell sorting.

The purpose of our study was to isolate nearly pure populations of viable round spermatids from mouse testicular cell populations by flow cytometry followed by cell sorting. We proposed a flow cytometric analysis on the basis of two parameters: cell size (forward scatter) and cell density (side scatter) related to the light diffracted by each cell passing through the laser field. Our method consisted of (i) the enrichment in spermatogenic cell population using centrifugation on a discontinuous Percoll gradient, (ii) the localization of round spermatids by flow cytometry and (iii) their isolation by cell sorting. Moreover, to characterize sorted cells, their capacity to express spermatid-specific genes was tested by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis.

Materials and methods

Cell preparation

C57BL/6 (black) male mice 4–8 weeks old were killed by cervical dislocation. The testes were surgically removed and placed in PBS

(Dulbecco's phosphate-buffered saline) at room temperature. The testes of one mouse were sufficient for each experiment. Under a dissecting microscope, the tunica albuginea and the remaining blood vessels on the surface of seminiferous tubules were removed with forceps. The tubule masses were rinsed twice in PBS, minced into small pieces using a pair of fine scissors then placed in 1 ml PBS at room temperature. Repeated pipettings for 1 min allowed spermatogenic cells to release into the medium. The resulting suspension was stored for 15 min at room temperature to allow the sedimentation of the remaining large fragments of intact tubules. The supernatant was centrifuged at 600 g for 5 min and the pellet was resuspended in 1 ml PBS.

Enrichment of spermatogenic cells

To increase the proportion of germ cells before flow cytometry analysis and cell sorting, the cell suspension was centrifuged on a discontinuous Percoll gradient prepared as follows. An iso-osmotic solution containing 90% Percoll was obtained by mixing 9 volumes of Percoll with 1 volume of 10-fold concentrated PBS. This 90% Percoll was used to prepare 45, 30, 22 and 15% Percoll solutions by dilution with PBS and a gradient was obtained by placing 1 ml of each Percoll solution carefully in a 15 ml conical tube before addition of the sample containing germ cells. After centrifugation (600 g, 25 min), cell populations recovered in the Percoll fractions were analysed by flow cytometry and we observed that spermatogenic cells were mostly recovered in the 22% Percoll fraction.

Preparation of control cells

Populations of mouse epididymal spermatozoa and lymphoid cells were analysed with the flow cytometer and compared with spermatogenic cells. The epididymides were minced in small pieces in 1 ml PBS placed in a Petri dish and incubated for 15 min at room temperature. Spermatozoa were recovered from the medium surrounding the epididymis fragments. Lymphoid cells were recovered from mouse spleen, homogenized with a Potter grinder and diluted with 0.5 ml PBS. The suspension was centrifuged for 15 min at 1200 g on a discontinuous (50, 65 and 85%) gradient of Percoll (Bijsterbosch and Klaus, 1986). The fraction at the 65–85% interface was free of erythrocytes and dead cells (Bijsterbosch and Klaus, 1986).

Flow cytometric analysis and cell sorting

The cell size (forward angle light scatter, FSC) and the cell density (90° light scatter, SSC) were simultaneously measured with either a FACSCalibur or a FACScan flow cytometer (Becton Dickinson, San José, CA, USA) both equipped with argon laser (power: 15 mW, wavelength: 488 nm). Cell sorting was performed only on the FACSCalibur equipped with a cell sorting system. These two flow cytometers were driven with the Cellquest software (Becton Dickinson). Instrument settings were adjusted so as that all events (cells and debris) were observed in the dot plot diagram. Resolution was of 1024 channels. FSC detector and SSC photodiode were set in linear mode and the light signal was divided by 10 (voltage: E-01) for FSC and was amplified (voltage: 367) for SSC. Some cellular and non-cellular debris were excluded by thresholding on the SSC parameter excluding particles below channel 54. Ten thousand events were analysed per sample.

An area (R1) delimiting the region containing the cell population to be sorted was drawn on the dot plot diagram with the use of the polygonal region tool of the Cellquest software (Figure 2B). The sheath fluid was a modified PBS medium (FASCFLOW) commercialized by Becton Dickinson. The cells were carried along slowly (flow rate of 12 µl/min) into the sheath fluid from the sample tube to the cell sorter. Cell sorting was programmed to accept analysed fractions

containing only selected cells (exclusion mode). The cells were strongly diluted in the flow medium during their transport into the flow cytometer and during sorting and the sorted cells were recovered in 50 ml culture tubes previously coated with bovine serum albumin [PBS plus 4% bovine serum albumin (BSA), 24 h at 4°C]. BSA concentration (~4%) must be maintained during the entire cell sorting procedure to limit the adhesion of spermatids to the surface of the plastic tubes.

Assessment of round spermatid viability after cell sorting

The viability of round spermatids after cell sorting was determined using the trypan blue exclusion test (Talbot and Chacon, 1981). The technical procedure used in our study was that described by Aslam *et al.* (1998) to assess the viability of human spermatogenic cells.

RT-PCR analysis

PolyA+ mRNA were isolated from sorted cells (R1 area) using the mRNA capture Kit (Boehringer Mannheim, Mannheim, Germany) according to the information supplied by the manufacturer. Briefly, after cell lysis and homogenization of the cells, mRNA were hybridized with a biotin-labelled oligo (dT)₂₀ probe. PolyA+ mRNA were immobilized in a streptavidin-coated PCR tube. After gentle washing, the captured mRNA could be used directly for reverse transcription and amplification in an RT-PCR assay.

One step reverse transcription and PCR amplification were carried out using the Titan RT-PCR system from Boehringer Mannheim, according to the manufacturer's instructions. The one-step reaction system used Avian Myeloblastosis Virus (AMV) for first strand synthesis and a mix which consisted of Taq DNA polymerase and Pwo DNA polymerase for the PCR part and a single optimized RT-PCR buffer. Briefly, two separate mixes were added to the PolyA+ mRNA-containing PCR tube in a total volume of 50 µl. Master mix 1 contained 0.2 µM of each deoxyribonucleoside 5'-triphosphate, 5 mM dithiothreitol, 10 U RNase inhibitor, 10 pmole of each of the sense and antisense primers and sterile water. Master mix 2 contained 10 µl RT-PCR buffer (5×) with Mg²⁺ (1.5 mM of MgCl₂), 1 µl enzyme mix (AMV+ Taq DNA polymerase and Pwo DNA polymerase) and sterile water.

The sample was placed in the Perkin Elmer Gene Amp 2400 Thermocycler equilibrated at 50°C and incubated for 30 min. The conditions for PCR were: initial denaturation for 2 min at 94°C followed by 10 cycles of 0.5 min denaturation at 94°C, annealing at x°C (depending on the primer couples, see Table I) for 0.5 min and elongation at 68°C for 45 s, plus 30 cycles with the same parameters except that the annealing time increased by 5 s per cycle, with a final step at 68°C for 7 min.

In an attempt to increase the sensitivity for the detection of *SP-10*, *c-kit* and hypoxanthine phosphoribosyltransferase gene (*HPRT*) transcripts, 2.5 µl of cDNA obtained from the first amplification served as template for a second DNA amplification reaction, using inner nested primers (Table I). The nested PCR conditions were: 40 cycles of 0.5 min denaturation at 94°C, annealing at x°C (depending on the primer couples, see Table I) for 0.5 min, elongation at 72°C for 0.5 min, with a final step at 72°C for 10 min, using AmpliTaq Gold™ (Perkin Elmer) DNA polymerase.

A simultaneous reaction in which reverse transcription enzyme was heat inhibited (for 5 min at 94°C) prior to the polymerase chain reaction was run as a control for the presence of DNA. Amplification of cDNA from whole testis served as positive control. For all amplifications, negative controls (water only) were included (data not shown). Primers for the amplification of the ubiquitous *HPRT* were used as a control for the synthesis of cDNA. Primer sequences for

Table I. Primers used in the reverse transcriptase–polymerase chain reaction assays

Gene	Primer sequences 5'–3' direction	Position	Annealing temperature (°C)	Product size (bp)		References
				cDNA	gDNA	
<i>HPRT</i>	CCTGCTGGATTACATTAAGCACTG	318–342	51	352	>352	Konecki <i>et al.</i> , 1982
outer	GTCAAGGGCATATCCAACAACAAAC	445–669				
<i>HPRT</i>	TAGTGATAGATCCATTCTATGACTG	351–376	50	292	>352	Konecki <i>et al.</i> , 1982
nested	GTCTGGAATTTCAAATCCAACAACACTG	620–642				
<i>PRM2</i>	CGTACC GAATGAGGAGCCCCAGTG	106–130	55	318	423	Johnson <i>et al.</i> , 1988
outer	TTAGTGATGGTGCCTCCTACATTTC	503–528				
<i>PRM2</i>	GGCCACCACCACCACAGACACAGGCG	235–260	60	156	261	Johnson <i>et al.</i> , 1988
nested	GCATCTCCTCCTCCTCGGGATCTTC	470–495				
<i>c-kit</i>	CTGGTGGTTCAGAGTTCCATAGAC	1451–1474	61	389	–	Qiu <i>et al.</i> , 1988
outer	TCAACGACCTTCCCGAAGGCACCA	1816–1839				
<i>c-kit</i>	GCGTCTTCCGGCACAACGGCACGG	1479–1502	55	294	–	Qiu <i>et al.</i> , 1988
nested	CCCATTGTGATCATAAGGAAGTTGCG	1746–1772				
<i>SP-10</i>	GGAGCACCACCAGTTCAGCCTGAGG	113–138	55	742	–	Liu <i>et al.</i> , 1992
outer	GCATCTAGACCTTGTTCAGAGAGG	830–854				
<i>SP-10</i>	GCATGCTGTAGCTGAACATTCTGCAGG	295–321	55	404	–	Liu <i>et al.</i> , 1992
nested	GCTGGGAGTTTTGAGTGGTGCATACTCC	671–698				

PRM2 = protamine 2 gene; *HPRT* = hypoxanthine phosphoribosyltransferase gene.

HPRT, *c-kit*, *SP-10* and *PRM2* annealing temperature and sizes of PCR products are shown in Table I.

The sensitivity of the nested PCR was evaluated by using cDNA from one, 10 and 50 sorted cells. Cells were collected under an inverted microscope ($\times 400$) with a fine pipette with an inner diameter of 50 μm . The PCR products (10 μl of each) were analysed on 2% agarose gels stained with ethidium bromide and molecular sizes were determined with the molecular weight marker λ X 174 *Hae* Digest (Sigma, Saint Quentin Fallavier, France).

Statistical analysis

Data were analysed using Statview software. Means (\pm SEM) were compared using Student's *t*-test.

Results

Enrichment in spermatogenic cells and cell sorting

In the suspensions, spermatozoa, elongating and round spermatids and primary pachytene spermatocytes were the more frequently observed cells (Figure 1). The dot plot diagrams obtained after Percoll gradient centrifugation (Figure 2B) showed that the cell population from the R1 area increased (14.6 versus 6.2%, $P < 0.005$) compared to the dot plot diagrams obtained without enrichment (Figure 2A). The 2-fold increase of the number of spermatid cells by enrichment optimizes conditions for cell recovery, i.e. reduction in time of the cell sorting procedure and decrease in volume of medium recovered after cell sorting.

According to dot plot diagram (Figure 2C) and optical microscope analysis (Figure 1D) R1 cells were very homogeneous in size ($\sim 11 \mu\text{m}$) and all revealed the cytological aspect of round spermatids: they were round-shaped with smooth outline and their nucleus contained one slightly eccentric nucleolus. A bright area and a dense nucleolus-like mass (probably an acrosomic granule) were adjacent to the nucleus in the cytoplasm (Figure 1D). When sorted cells from the R1 area were analysed again by the flow cytometer (Figure 2C), a well-defined area of spermatids were easily recognizable on the dot plot diagram (Figure 2C). Moreover, microscope

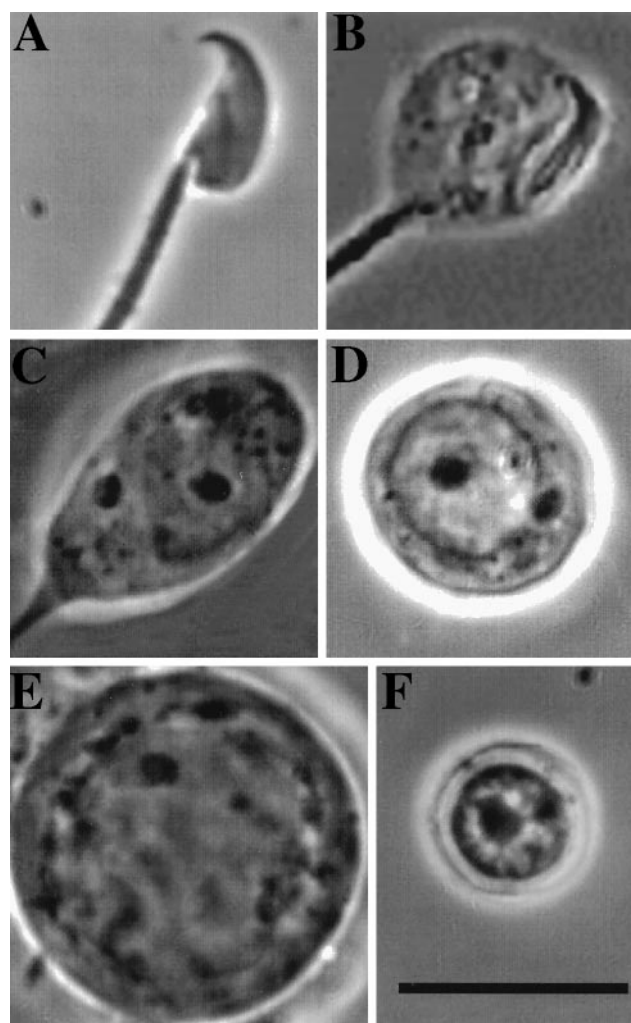


Figure 1. Phase-contrast photomicrographs of the mouse spermatogenic cells mostly observed in the 22% Percoll fraction. Mouse spermatozoon (A); late elongating spermatid (B); elongating spermatid (C); round spermatid (D); primary pachytene spermatocyte (E); lymphoid cell (F). Scale bar = 10 μm .

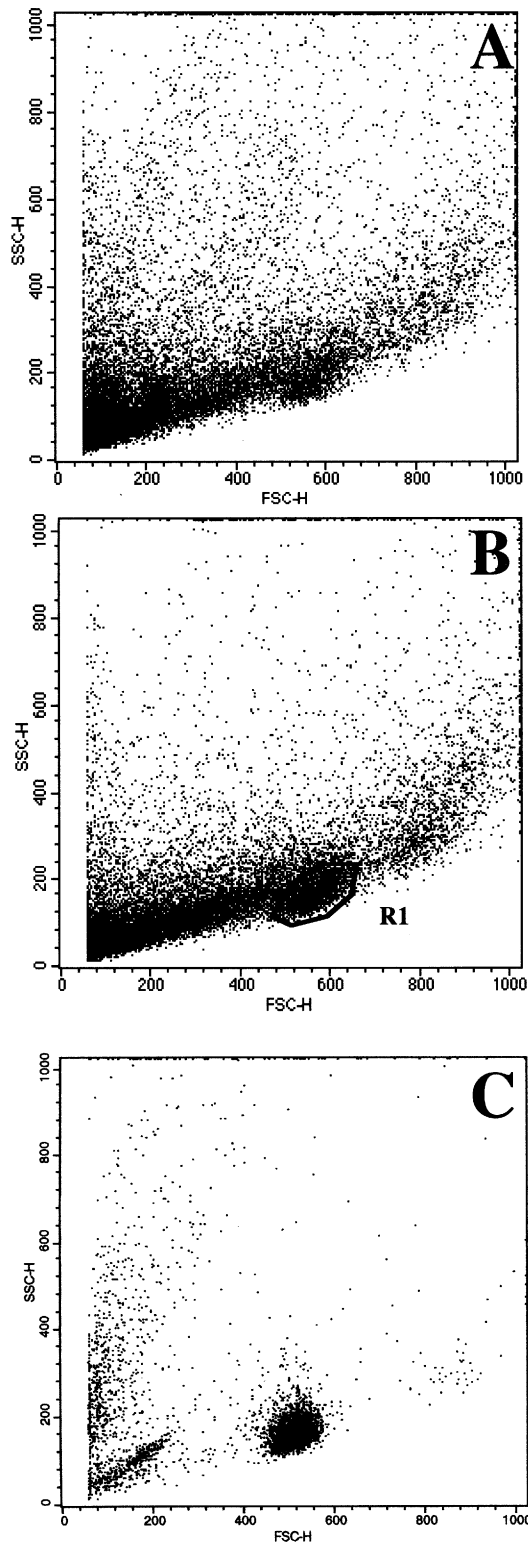


Figure 2. Flow cytometry analysis of mouse testis population without (A) and following (B) Percoll gradient centrifugation and after isolation of R1 cell population by cell sorting (C). Swollen and/or altered round spermatids were observed in the vicinity of the R1 area (C), probably due to the cell re-exposure to the laser beam. The cell size (FSC, forward angle light scatter) and the cell density (SSC, 90° light scatter) were simultaneously measured by the flow cytometer. x and y axes are arbitrary scales.

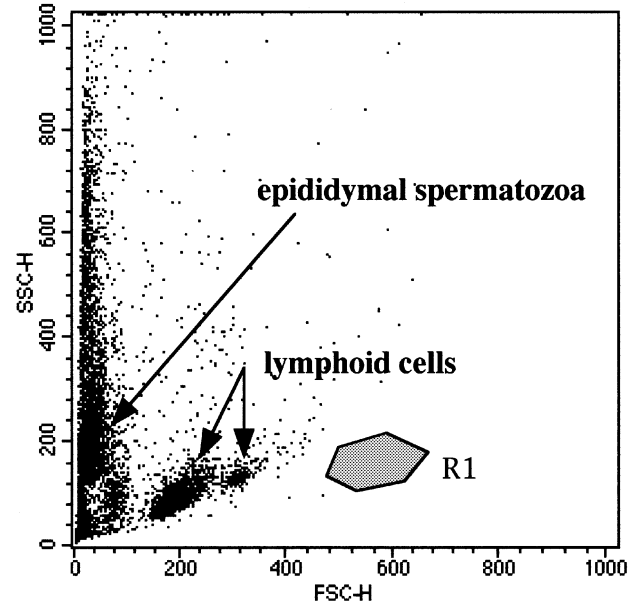


Figure 3. Mouse epididymal spermatozoa and mouse lymphoid cells analysed by flow cytometry and compared with spermatogenic cell suspension (R1 area). FSC = forward angle light scatter; SSC = 90° light scatter.

analysis showed that the sorted cells were almost all round spermatids and >99% of them retained their viability as assessed by the trypan blue exclusion test.

Mouse epididymal spermatozoa and spleen lymphocytes were analysed with the same flow cytometric parameters. Dot plot diagrams showed that the arbitrary sizes of epididymal spermatozoa and lymphocytes were between 20 and 100 and between 100 and 350 on the FSC axis, respectively (Figure 3), whereas the R1 area was localized between 400 and 600 on the FSC axis (Figure 2B and C). Lymphoid cells were characterized by a smaller size ($6.8 \pm 0.2 \mu\text{m}$), a larger nucleocytoplasmic ratio so that a slight and regular zone of cytoplasm surrounding the nucleus was observed (Figure 1F).

Characterization of sorted cell by RT-PCR

Pellets of 3000 cells corresponding to fraction R1 from the FACSCalibur cell sorting procedure were analysed for transcripts of either *PRM2*, *SP-10*, *c-kit* or *HPRT* (Figure 4). The PCR results showed that *PRM2* and *SP-10* genes are expressed in the cell population tested. Even when performing a nested PCR assay, the *c-kit* signal was absent in this population (R1) as expected. Two of the PCR primers used amplified the mRNA sequence encoding the transmembrane portion of the *c-kit* protein, which is missing in the alternative *c-kit* transcript expressed in round spermatids (Rossi *et al.*, 1992). A weak *HPRT* signal was obtained only when nested PCR was performed. The mouse *HPRT* gene maps to the X chromosome, which is inactivated at meiosis in the male germ cell line (Monesi *et al.*, 1978). Both *HPRT* and *c-kit* transcripts were detected when starting with mRNA from whole testis. To exclude the possibility that the specific signals were derived from contaminating DNA, all samples were tested by PCR without prior reverse transcription. In addition, the primers for *PRM2* and *HPRT* were designed to span one or more introns

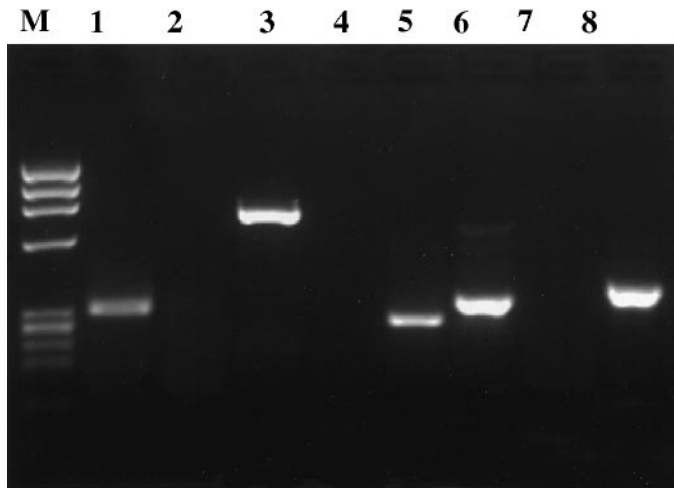


Figure 4. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of R1 population for *PRM2*, *SP-10*, *c-kit* and *HPRT* expression. RT–PCR was performed on polyA⁺ mRNA from 3000 cells. Lanes 1 and 2 were RT⁺ and RT[–] samples with *PRM2* primers. Lanes 3 and 4 were RT⁺ and RT[–] samples with *SP-10* primers. Lanes 5 and 7 were nested RT–PCR with *HPRT* and *c-kit* inner primers, respectively. Lanes 6 and 8 were positive control with polyA⁺ mRNA from whole testis for *HPRT* and *c-kit* expression, respectively (outer primers).

(Table I) such that the cDNA-derived products could be distinguished from possible amplified genomic products by size on an agarose gel. No products deriving from genomic DNA templates were obtained.

The PCR assay was sufficiently sensitive to detect the presence of *SP-10* in 10 cells, whereas a nested PCR was necessary to demonstrate the expected 404 bp fragment in a single cell (data not shown).

No transcripts for *PRM2* or *SP-10* were detected with the nested PCR procedure when utilizing mRNA from 30 000 mouse spleen lymphocytes obtained after the cell sorting procedure. On the contrary, transcripts of *HPRT* were highly expressed (data not shown) as a strong signal was obtained using only the outer primers in a one round PCR amplification reaction.

Discussion

We have demonstrated that viable round spermatids can be isolated from testis by flow cytometry and cell sorting. Microscope analysis showed that the sorted cells from the R1 area were almost all round spermatids. More than 99% of sorted round spermatids were viable after cell sorting. Round spermatids were easily recognizable as a large and well-defined area (R1) on the dot plot diagram from both entire or enriched spermatogenic cell populations analysed by flow cytometry. Cells from the 22% Percoll fraction were mostly round spermatids, spermatozoa and primary pachytene spermatocytes, whereas elongating spermatids were rarely observed. Moreover, the number of round spermatids was increased 2-fold from the cell sorting procedure following Percoll gradient centrifugation, which allowed an enrichment in spermatogenic cells. Sorted R1 cells were very homogeneous in size (~11 μ m) and had all a cytological aspect characteristic of round spermatids.

Several authors (Kimura and Yanagimachi, 1995a,b; Sasagawa and Yanagimachi, 1997; Sasagawa *et al.*, 1998) have succinctly described the morphology and size of live mouse round spermatids. They are ~10 μ m in diameter (ranging from 9 to 11 μ m) and have a round shape with a smooth outline. Their round nucleus contains a centrally located nucleolus. Under phase-contrast microscopy, we observed in the cytoplasm the presence of a bright area and a dense nucleolus-like mass, both adjacent to the nucleus as already described in the rabbit (Sofikitis *et al.*, 1994), bovine (Goto *et al.*, 1996) and human spermatocyte (Shepherd *et al.*, 1981; Tesarik and Mendoza, 1996; Angelopoulos *et al.*, 1997; Antinori *et al.*, 1997). The bright area was previously described in human cells as either a developing acrosomal granule/vesicle (Tesarik and Mendoza, 1996) or a juxtannuclear annulate lamella (Shepherd *et al.*, 1993), whereas the dense nucleolus-like mass seems to be an acrosomal granule according to Sofikitis *et al.* (1997, in rabbit) and Shepherd *et al.* (1993, in human). Like round spermatids, lymphoid cells showed a round shape with a smooth outline. However, lymphoid cells, which are characterized by a larger nucleocytoplasmic ratio and a diameter of between 6 and 7 μ m, could not be confused with round spermatids.

To ascertain the maturation stage of the germ cells obtained by flow cytometry and cell sorting procedure, we looked for the expression of two genes, *PRM2* and *SP-10*, which are exclusively transcribed from the haploid genome (Mali *et al.*, 1989; Kurth *et al.*, 1991). *PRM2* is a small arginine-rich protein that replaces histones and transition proteins on the DNA during the later stages in spermatogenesis and its mRNA is first detectable in step-7 spermatids (Hecht, 1992) whereas *SP-10* is a marker for acrosome biogenesis (Kurth *et al.*, 1993). By RT–PCR analysis, we clearly demonstrated the expression of both genes in the R1 cell population, confirming that R1 is mainly composed of round spermatids.

The possible contamination of the R1 cell fraction with spermatogonia or lymphoid cells whose sizes are close to that of spermatids has been evaluated. The expression of the 5.5 kb *c-kit* transcript, which is specific premeiotic germ cells (Sorrentino *et al.*, 1991), was evaluated by RT–PCR on the R1 cell fraction to detect the presence of spermatogonia. The nested PCR assay was able to identify a single cell as demonstrated using *SP-10* primers. The absence of any amplified fragment of the expected size pointed to minor, if any, contamination with spermatogonia.

We observed that lymphoid cells express high levels of *HPRT*. On the contrary, a nested RT–PCR was necessary to detect a weak signal with *HPRT* mRNA from the R1 sorted population. These results are in agreement with those of Rossi *et al.* (1993) who did not find any *HPRT* expression in round spermatids. Moreover, the weak signal obtained after nested PCR could be due to the presence of residual *HPRT* mRNA, even though we cannot exclude a slight contamination with few other cells. Taken together these results clearly established that the R1 cell-sorted population was composed of round spermatids, and was highly homogeneous.

In our study, few sorted cells showed morphological alterations such as swelling associated with cell density changes or the entire loss of the cytoplasm. Our method for spermatogenic

cell recovery *per se* (seminiferous tubule dissection, repeated pipetting and Percoll gradient centrifugation) could have been damaging, but a possible harmful effect of the laser beam could also not be excluded. Several studies have shown that haematopoietic stem cells remain viable and retain their biological functions after flow cytometry and cell sorting procedures (Szilvassy *et al.*, 1989; McAlister *et al.*, 1990; Lebkowski *et al.*, 1990; Chen *et al.*, 1995; Sasaki *et al.*, 1995; Leemhuis *et al.*, 1996). Their medical use as a haematopoietic graft has been suggested (Chen *et al.*, 1995; Sasaki *et al.*, 1995). Moreover, when sorted round spermatids were microinjected into mouse oocytes, normal fertilization occurred (as assessed by the appearance of two pronuclei) and 2-cell embryos were obtained from 30% of the oocytes (our unpublished data).

In conclusion, our results clearly established that a highly homogeneous population of viable round spermatids could be isolated from testis by flow cytometry followed by cell sorting. The procedure is fast and allows the purification of spermatids from a low number of crude testicular cells. Moreover, no fluorochrome was used in our study to identify cells. This is a definitive advantage in the case of in-vitro studies of sorted cells or of microinjection of round spermatids into oocytes compared to technical procedures using either immunoglobulins, conjugated lectins or DNA intercalants to identify spermatids. However, more studies are needed to assess the risks of flow cytometry and cell sorting to round spermatid viability and integrity, particularly if this technique is to be used for human spermatid isolation (Aslam *et al.*, 1998).

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