

## Flow cytometry isolation and reverse transcriptase–polymerase chain reaction characterization of human round spermatids in infertile patients

Ahmed Ziyat<sup>1</sup>, Bruno Lassalle<sup>1</sup>, Jacques Testart<sup>1,2</sup>, Pascal Briot<sup>2</sup>, Edouard Amar<sup>2</sup>, Catherine Finaz<sup>1</sup> and Annick Lefèvre<sup>1,3</sup>

<sup>1</sup>Unité Maturation Gamétique et Fécondation, INSERM U 355 and Institut Fédératif de Recherche sur les Cytokines, IFR 13, 32 rue des Carnets, 92140 Clamart and <sup>2</sup>Centre de Médecine de la Procréation, Hôpital Américain, 63, Boulevard Victor Hugo, 92202 Neuilly, France

<sup>3</sup>To whom correspondence should be addressed at: INSERM unité 355, 32 rue des Carnets, 92140 Clamart, France

**Flow cytometry coupled to cell sorting is proposed as a method to isolate round spermatids from testicular biopsies in obstructive azoospermic patients. The cells were separated on the basis of their size and density only. We obtained homogenous populations of alive round spermatids free of lymphocytes and diploid germ cells. The detection of protamine 1 gene (*PRM1*) and *PRM2* expression in the sorted cells proves that these cells are round spermatids. On the contrary, neither the expression of *CD3-δ*, which is specific to lymphoid cells, nor that of *MAGE1*, which has been demonstrated in diploid germ cells, could be observed in the round spermatid population even after using a nested polymerase chain reaction (PCR) assay. The flow cytometry procedure failed to isolate round spermatids from ejaculates in non-obstructive azoospermic patients. In >39 ejaculates tested by reverse transcriptase–PCR, only nine revealed the presence of some round spermatids, as demonstrated by the expression of *PRM1*. However, these round spermatids did not express *PRM2*.**

*Key words:* flow cytometry/obstructive azoospermia/protamine/round spermatids

### Introduction

The round spermatid is the result of the second meiotic division in males. It is the youngest male germ cell with a set of haploid chromosomes that differentiates into a spermatozoon to acquire its fertilizing ability. Sofikitis *et al.* (1994) were the first to report a completed pregnancy in mammals achieved from the injection of a round spermatid into a rabbit oocyte. Ogura *et al.* (1994) and Kimura *et al.* (1995) obtained the birth of normal fertile mice of both sexes after electrofusion or injection of mouse oocytes with round spermatids respectively. Although the success rates were very low, these experiments showed that there is no genetic barrier to fertilization by round spermatids. Studies on electrical oocyte activation before round spermatid nuclear injection showed that a simple technical amelioration alone have led to a considerably improved success

rate (Sofikitis *et al.*, 1996). The authors of these pioneering experiments concluded that the nucleus of the round spermatid is genetically ready to participate in the normal fertilization process.

These results have offered new prospects for the treatment of male infertility due to defective spermiogenesis in humans. Vanderzwalmen *et al.* (1995) were the first to succeed in fertilizing a human oocyte by a late-stage spermatid (obtained from testicular biopsy) that was microinjected. All oocytes cleaved further to 4-cell embryos. Fishel *et al.* (1995) reported the implantation of such embryos after uterine transfer. The first birth of a healthy child after round spermatid injection into human oocytes (Tesarik *et al.*, 1995) confirmed the feasibility of this novel approach in the treatment of non-obstructive azoospermia. In another paper, Tesarik *et al.* (1996) provided a complete documentation of the series of 11 cases using spermatids retrieved from ejaculated semen. In four cases, elongated spermatids were used, whereas in the other seven cases, round spermatids were injected. Two pregnancies were achieved, resulting in the birth of two normal boys. These first results have encouraged many clinics to introduce in their intracytoplasmic sperm injection (ICSI) programme the treatment of azoospermic patients using spermatids. Thus, Araki *et al.* (1997) reported three cases of successful paternity, achieved by intracytoplasmic injection of late spermatids leading to the birth of four healthy boys and girls. Several other pregnancies and births were then obtained after injection of either elongated or round spermatids, both retrieved from testicular biopsies (Antinori *et al.*, 1997a; Vanderzwalmen *et al.*, 1997; Kahraman *et al.*, 1998; Sofikitis *et al.*, 1998).

Currently, it is not clear which source is preferable to obtain spermatids in testicular or ejaculated samples. If the testis is the source of spermatids, several biopsies may be necessary before a pregnancy could be finally achieved even when the recovered spermatids are frozen. One successful fertilization and pregnancy has been obtained after injection of freeze–thawed round spermatids (Antinori *et al.*, 1997b). On the other hand, Tesarik *et al.* (1996) advocate the use of spermatids recovered from the ejaculate, as the least invasive approach sparing the residual foci of spermatogenesis eventually present in the testis. Antinori *et al.* (1997a,b) reported the complete absence of spermatids after the extensive examination of the ejaculate of all the patients entering their spermatid ICSI programme.

The main point clinicians are very much concerned with is the difficulty of identifying with certainty round spermatids even under a Hoffman modulation contrast microscope (Vanderzwalmen *et al.*, 1997). As reported by Yamanaka *et al.* (1997), the qualitative criteria retained, i.e. a regular zone of

cytoplasm surrounding a round nucleus and a developing acrosome structure, are highly susceptible of intra- and extra-observer variations. It may result in recovery of round cells rather than round spermatids, and this may suggest one reason for the low fertilization rate with round spermatids.

Therefore, the present study has been designed to answer the following puzzling questions: are we able to propose a fast and reliable procedure to isolate pure populations of viable well-characterized round spermatids, and are ejaculates from non-obstructive azoospermic patients a possible alternative source of round spermatids? We have used flow cytometry/cell sorting to purify human round spermatids from testicular biopsies and ejaculates from infertile patients, previously optimized with mouse spermatids (Lassalle *et al.*, 1999). The presence of round spermatids has been demonstrated by the expression of protamine 1 gene (*PRM1*) and *PRM2*. During the time we were writing this article, Aslam *et al.* (1998) have published a comparative study between velocity sedimentation under unit gravity and fluorescent activated cell sorting but they have identified the cells only on the basis of their morphological characteristics.

## Materials and methods

### Patients

A total of 51 patients was involved in this study. They were consulted and informed about the nature of this study. All gave their consent.

### Testicular biopsies

Testicular tissues used for round spermatid isolation were obtained from patients undergoing testicular biopsy retrieval for infertility treatment by ICSI. The seven cases retained for the study were obstructive azoospermia, showing normal spermatogenesis with all types of germ cells, motile spermatozoa included. Biopsies were performed through standard open surgical technique and the retrieved tissue was immersed and rinsed twice in PBS (Dulbecco's phosphate-buffered saline; Sigma; Saint Quentin, Fallavier, France), minced into small pieces using a pair of fine scissors and placed in 1 ml PBS at room temperature. Continuous pipetting for 1 min enabled spermatogenic cells to be released into the medium. The resulting suspension was left 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. To increase the proportion of round spermatids, prior to flow cytometry and cell sorting, the cell suspension was centrifuged on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient (45, 30, 22, and 15%) as previously described (Lassalle *et al.*, 1999). After centrifugation, the 22% Percoll fraction, where most of the round spermatids sedimented, was analysed by flow cytometry.

### Ejaculate samples

Ejaculates from 44 patients with non-obstructive azoospermia were analysed in order to look for spermatids. The mean age of these patients was 33.8 years (range, 26–48 years). Chromosome analysis was performed on 28 patients and all their karyotypes were normal (46,XY). Lysis of erythrocytes was performed as previously described (Sofikitis *et al.*, 1994). The cells were washed twice with PBS, resuspended in 1 ml of PBS and analysed via flow cytometry (14 of them) and/or kept at –80°C for further mRNA extraction and RT-PCR treatment.

### Lymphoid cells recovery

Haemolysed human blood was centrifuged over a discontinuous gradient (90, 70, 45 and 22%) of Percoll and lymphoid cells were recovered in the 70% Percoll fractions as already described by Kolb *et al.* (1993). Monoclonal mouse anti-human CD 45 fluorescein isothiocyanate (FITC)-conjugated antibody (PharMingen, San Diego, USA) was used (dilution: 1/100 in PBS; incubation 30 min at 37°C) to confirm the presence of lymphoid cells.

### Microscope observations and cell size measurement

Ten microlitres of the cell suspensions were placed between a slide and coverslip. Small amounts of vaseline were applied on the slide at four points around the droplet containing cells before the coverslip was placed on top and pressed slightly to limit cell crushing. The diameter of cells was measured with  $\times 1000$  magnification using the micrometer in one eyepiece of a phase-contrast microscope. One graduation of the ocular micrometric scale was estimated to 0.97  $\mu\text{m}$ .

### Flow cytometric analysis and cell sorting

Cell analysis and sorting were performed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a cell sorting system. The cell size (forward angle light scatter, FSC) and the cell density (90° light scatter, SSC) were simultaneously measured. The FACScalibur instrument settings were adjusted as already described by Lassalle *et al.* (1999). Ten thousand events were analysed per sample. The sorted cells were recovered in 50 ml culture tubes previously coated with bovine serum albumin or fetal calf serum (PBS plus 4% BSA or FCS, 24 h at 4°C). BSA or FCS concentration (~4%) must be maintained during the entire cell sorting procedure to limit the adhesion of spermatids on the surface of the plastic tube.

### Assessment of viability

After cell sorting, viability of round spermatids was determined by the Trypan Blue exclusion test (Talbot and Chacon, 1981).

### RT-PCR analysis

#### Extraction of mRNA

PolyA+ mRNA were isolated from either all ejaculated cells or FACScalibur purified round spermatids originating from biopsies, using the mRNA capture kit (Boehringer Mannheim), as previously described (Lassalle *et al.*, 1999).

#### One-step reverse transcription and PCR amplification

Reverse transcription and PCR were done in one step using the Titan RT-PCR system from Boehringer Mannheim, according to the manufacturer information as previously described (Lassalle *et al.*, 1999). Outer and nested primer sequences for glucose-6-phosphate dehydrogenase (*G6PDH*), protamine 1 (*PRM1*), protamine 2 (*PRM2*), *MAGE-1* and *CD3- $\delta$* , annealing temperatures and sizes of PCR products are shown in Table I.

In an attempt to increase the sensitivity for the detection of these gene transcripts, 2.5  $\mu\text{l}$  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 as previously described (Lassalle *et al.*, 1999).

A simultaneous reaction in which reverse transcriptase enzyme was heat-inhibited (for 5 min at 94°C) prior to the PCR was run as a control for the presence of DNA. Amplification of cDNA from whole testis served as a positive control. For all amplifications, negative controls (water only) were included (data not shown). Primers for the amplification of the ubiquitous *G6PDH* gene were used as a control for the synthesis of cDNA. The PCR products

**Table I.** Details of primers

Gene	Primer sequences 5'–3' direction	Position	Annealing temperature (°C)	Product size (bp)		References
				cDNA	gDNA	
<i>PRM1</i> outer	a. GCCAGGTACAGATGCTGTCGCAG	4–26	60	150	241	Domenjoud <i>et al.</i> , 1990
	b. GTGTCTTCTACATCTCGGTCTG	223–244				
<i>PRM1</i> nested	d. TACCTGGGGCGGCAGCACC	204–222	60	128	219	
<i>PRM2</i> outer	a. GTCCGATACCGCGTGAGGAGCCTG	4–27	60	301	464	Domenjoud <i>et al.</i> , 1990
	b. GCCTTCTGCATGTTCTTCTCTGG	443–467				
<i>PRM2</i> nested	c. CGCTCGCACGAGGTGTACAGGCAG	34–57	60	271	434	
<i>G6PDH</i> outer	a. GGTGAGGCTGCAGTTCATGATGTGG	16753–16778	60	338	548	Chen <i>et al.</i> , 1991
	b. GGATGGGCTTGGGCTTCTGCAGCTC	17275–17299				
<i>G6PDH</i> nested	c. CCAAGATGATGACCAAGAAGCCGGG	16854–16878	60	207	417	
	d. GGTGCAGCAGTGGGGTGAATAACGC	17244–17269				
<i>CD3-δ</i> outer	a. GGGAACGGTGGGAACACTGC	92–111	55	225	1054	Van Den Elsen <i>et al.</i> , 1986
	b. AAAGCAAGGAGCAGAGTGGC	764–783				
<i>CD3-δ</i> nested	c. GACTGGACCTGGGAAAACGC	127–146	60	189	656	
<i>MAGE1</i> outer	a. CAGGGGACAGGCCAACCAGAGG	418–440	60	381	456	Van Der Bruggen <i>et al.</i> , 1991
	b. CGGCTGCTGGAACCCTCACTGGG	851–873				
<i>MAGE1</i> nested	c. GAGCCACAGAGGAGCACCAGG	453–476	60	213	288	
	d. CAGAGGAGAGGAGGAGGAGGTGG	717–740				

a. and c. are sense primers, b. and d. are antisense primers.

(10 µl of each) were analysed on 2% agarose gel stained with ethidium bromide and molecular sizes were determined with the molecular weight marker *f* X 174 *Hae* Digest (Sigma).

#### Statistical analysis

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

## Results

### Testicular biopsies from obstructive azoospermia

#### Isolation of round spermatids

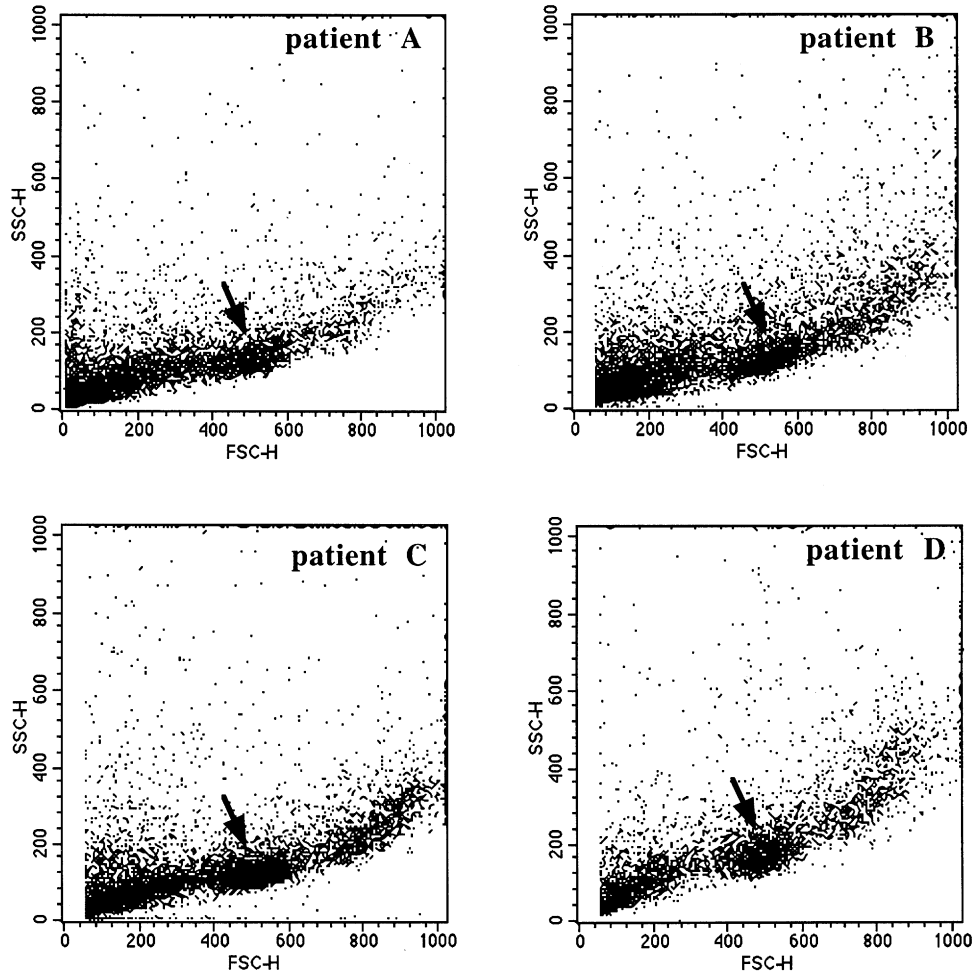
After manual mincing and moderate pipetting, the cell suspension obtained from the testicular biopsies of obstructive azoospermic patients consisted mainly of spermatozoa, elongating and round spermatids and primary spermatocytes. This cell suspension was first enriched 2-fold in round spermatids by centrifugation on a discontinuous Percoll gradient. Round spermatids sedimented mainly in the 22% fraction according to the results of Meistrich *et al.* (1981) in the rat and Lassalle *et al.* (1999) in the mouse, while lymphocytes were found preferentially in the 70% fraction as expected (Kolb *et al.*, 1993).

When the 22% Percoll fraction was subjected to flow cytometry coupled with cell sorting, round spermatids were recovered as a homogeneous population in a delimited area localized between 400 and 600 on the FSC axis (arbitrary units). The dot plot diagrams were very similar from one patient to another (Figure 1), except that the round spermatid populations were more or less abundant. Lymphocytes obtained from human blood, which served as control, were found in a well-defined area at ~200 on the FSC axis (Figure 2A). This

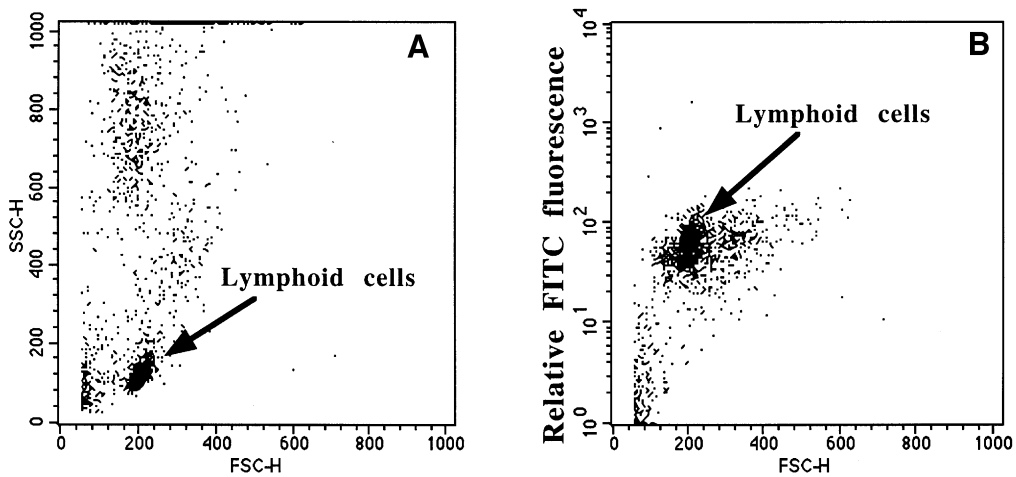
was corroborated by the use of a mouse monoclonal anti-human CD45 antibody conjugated with FITC (Figure 2B). Round spermatids and lymphocytes were sorted in distinct areas as a consequence of their different sizes. Round spermatid populations were very homogeneous with an average size of  $10.6 \pm 0.5$  µm (Figure 3C). Typical characteristics of round spermatids were observed: a round shape with a smooth outline, a round nucleus showing a distinct eccentric nucleolus, a well-formed acrosomal cap, an acrosomal granule and a continuous zone of cytoplasm surrounding the nucleus. More than 99% of the sorted spermatids retained their viability as assessed by the Trypan Blue exclusion test. Figure 3 shows three stages of haploid cells observed under a phase-contrast microscope. Figure 3A shows an elongating spermatid from the 22% Percoll gradient fraction. Figure 3B and C represents round spermatids isolated by the FACScalibur procedure; on spermatid B the acrosomal cap is clearly visible, whereas on spermatid C the early acrosomal vesicle is present as a bright white spot. The lymphocytes were much smaller in size,  $7.1 \pm 0.7$  µm, and exhibited a larger nucleocytoplasmic ratio (Figure 3D).

#### RT-PCR characterization of the sorted cells

mRNA corresponding to 3000 cells of the purified round spermatid population were analysed for the presence of *PRM1* and *PRM2*, *MAGE1* and *CD3-δ* transcripts. All primers were designed to span one or more introns (Table I) so that the PCR products could be distinguished from possible genomic amplified products by size on an agarose gel. No products deriving from genomic DNA templates were obtained. In addition, experiments omitting the reverse transcription step confirmed the specificity of the products to mRNA. Blanks



**Figure 1.** Flow cytometric analysis of spermatogenic cell populations obtained from human testicular biopsies (four representative cases). The round spermatid population is easily recognizable on the dot plot diagram as indicated by arrows.

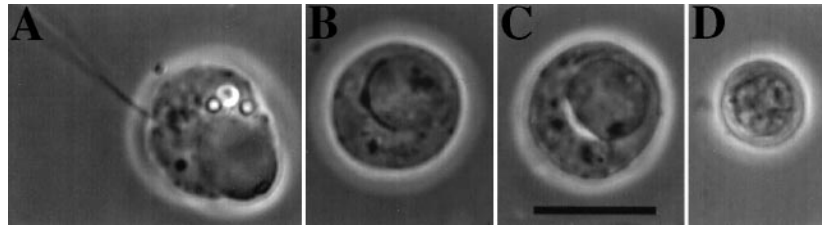


**Figure 2.** Flow cytometric analysis of lymphoid cells isolated from human blood. Lymphoid cells were stained with a mouse monoclonal anti-human CD45 antibody conjugated with fluorescein isothiocyanate (FITC), then analysed by flow cytometry on the basis of (A) FSC/SSC (cell size/cell density) and (B) FSC/FL1 (cell size/relative FITC fluorescence) parameters.

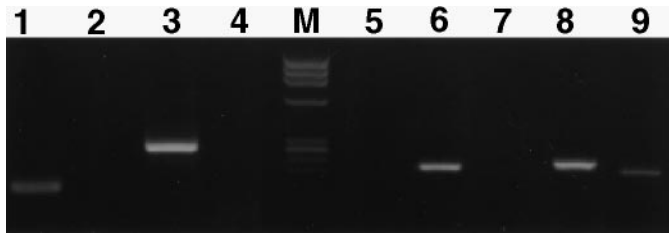
consisting of PCR reaction mixture without added template were always included to ensure absence of contamination.

Both *PRM1* and *PRM2* transcripts were detected in the

round spermatid population (Figure 4). mRNA isolated from a single cell gave a positive signal when performing a nested PCR assay with *PRM1* primers (14 independent experiments



**Figure 3.** Phase-contrast microscopy of human spermatid cells. (A) Elongating spermatid. (B) Round spermatid with a well-formed acrosomal cap. (C) Round spermatid showing an acrosomal vesicle. (D) Human lymphoid cell. Scale bar = 10  $\mu$ m.



**Figure 4.** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *PRM1*, *PRM2*, *MAGE1* and *CD3- $\delta$*  expression. RT-PCR was performed on poly A+ mRNA from 3000 round spermatids or lymphocytes and on 1  $\mu$ g of total mRNA from whole testis. Lane 1 and 2, RT+ and RT- round spermatid samples with *PRM1* primers. Lanes 3 and 4, RT+ and RT- round spermatid samples with *PRM2* primers. Lanes 5, RT+ round spermatid sample with *MAGE1* nested primers. Lane 6, RT+ whole testis sample with *MAGE1* nested primers. Lane 7, RT+ round spermatid sample with *CD3- $\delta$*  nested primers. Lanes 8 and 9, RT+ lymphocyte samples with *CD3- $\delta$*  outer and nested primers respectively. M, f *Hae* III marker. *PRM1* PCR product was 150 bp; *PRM2* PCR product was 301 bp; *MAGE1* nested PCR product was 213 bp; *CD3- $\delta$*  product was 225 bp; *CD3- $\delta$*  nested PCR product was 189 bp.

were done; data not shown). After a first round of PCR, mRNA representing *MAGE1*, which is expressed in spermatogonia and primary spermatocytes (Takahashi *et al.*, 1995), were detected in total testis, but not in the round spermatid population, even after performing a nested primer assay. As expected, transcripts for *CD3- $\delta$*  were detected in lymphocytes after one round of PCR, but not in round spermatids as confirmed by performing a second round of PCR with nested primers.

#### Ejaculates from non-obstructive azoospermia

Figure 5 shows the dot plot diagrams obtained when ejaculates of non-obstructive azoospermic patients were analysed by flow cytometry. For the 14 ejaculates tested, no spot appeared in the area where round spermatids were expected. Seven patients exhibited a spot of lymphocytes. Most of the ejaculates were heavily contaminated with bacteria and cell debris. This absence of spot in the area where round spermatids are expected was also observed when normospermic ejaculates were subjected to flow cytometry/cell sorting.

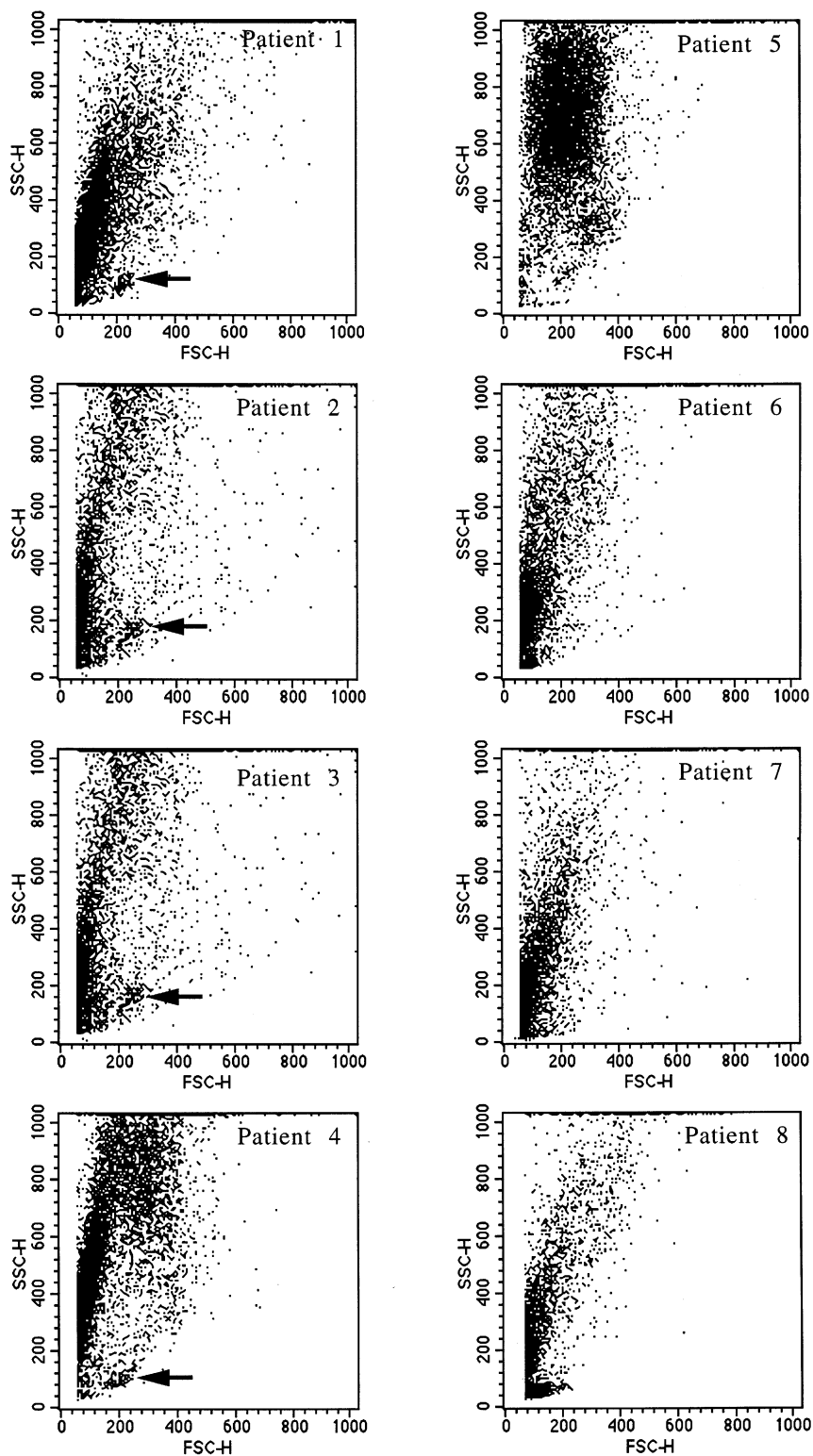
Thirty-nine crude ejaculates were analysed using a nested PCR protocol for the expression of *PRM1* and *PRM2*. Primers for the amplification of the ubiquitous *G6PDH* gene were used as a control for the synthesis of cDNA and the 207 bp fragment attended was generated in all the samples (Figure 6). Two

samples only showed the 150 bp fragment corresponding to the outer primers *PRM1* amplicon, while seven others were weakly positive after a second round of PCR with semi-nested primers. Only two of these positive samples in the semi-nested PCR assay belong to the group of 14 patients previously assayed with flow cytometry, the 12 others being negative. mRNA corresponding to *PRM2* was not detected in any sample, even after nested PCR (data not shown).

#### Discussion

When microinjected into mammalian oocytes, spermatids were able to fertilize and to produce live offspring (Ogura *et al.*, 1994; Sofikitis *et al.*, 1994; Kimura *et al.*, 1995). It has been suggested that spermatid injection may be the treatment of choice for patients having defective spermatogenesis (Edwards, 1994; Sofikitis *et al.*, 1994). In cases of azoospermia caused by testicular failure including Sertoli cell-only syndrome, maturation arrest, cryptorchid testicular atrophy or Klinefelter's syndrome, areas of some persisting spermatogenesis can be detected eventually after multiple testicular biopsies (Devroey *et al.*, 1995; Silber *et al.*, 1996). The few spermatozoa retrieved can result in normal pregnancy after ICSI (Silber *et al.*, 1996). However, in 40% of azoospermic men with germinal failure, no mature spermatozoa could be found, despite the presence of tubules with complete spermatogenesis in a previous biopsy for some of them. In fact, fertilization and pregnancies can be obtained using elongating and elongated spermatids with an acceptable implantation rate, as compared to conventional ICSI (Fishel *et al.*, 1995, 1997; Vanderzwalmen *et al.*, 1995, 1997; Antinori *et al.*, 1997a; Araki *et al.*, 1997; Kahraman *et al.*, 1998; Sofikitis *et al.*, 1998).

On the other hand, even though many attempts have been made, the capacity of round spermatids for achieving fertilization and pregnancy is disappointing, and the birth of healthy babies after ROSI (round spermatid injection) remains exceptional (Tesarik *et al.*, 1995; Vanderzwalmen *et al.*, 1997; Antinori *et al.*, 1997b; Kahraman *et al.*, 1998). The causes of such a relative failure are puzzling. It is generally agreed that it is very difficult to differentiate with certainty a round spermatid from the variety of 'round cells' present either in testicular biopsies or in ejaculates. The resulting confusion may account, in part, for the inefficiency of round spermatids to achieve pregnancy (Silber and Johnson, 1998). None of the techniques utilized so far have provided homogeneous populations of round spermatids. Tesarik and Mendoza (1996)

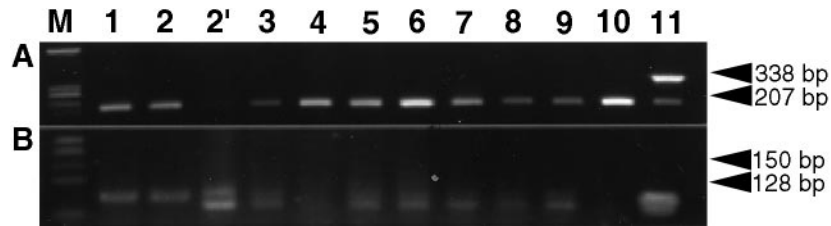


**Figure 5.** Flow cytometric analysis of ejaculate cells from eight representative non-obstructive azoospermic patients. No round spermatid spots could be visualized. Arrows indicate the lymphoid cell spots.

admitted that the round spermatid enriched fraction utilized in their ROSI programme contained only 1–5% spermatids.

Therefore, the development of a reliable technique for the isolation of round spermatids appears to be essential if microinjection is to be proposed as a new treatment for male

infertility. The aim of our work was to find a reliable method of obtaining populations of human round spermatids from either testicular biopsies or ejaculates, with the exclusion of other round cell types, via flow cytometry coupled to cell sorting.



**Figure 6.** Analysis of *PRMI* transcription in non-obstructive azoospermic patient ejaculates. Reverse transcriptase–polymerase chain reaction (RT–PCR) was performed on poly A+ mRNA from whole ejaculates. (A) Oligonucleotide primers for *G6PDH* were included in each analysis as a control for the presence of cDNA. Lanes 1–10, patient samples with *G6PDH* inner primers. Lane 11, whole testis sample with outer and nested *G6PDH* primers. *G6PDH* PCR product was 338 bp; nested *G6PDH* PCR product was 207 bp. (B) Lanes 1 and 2, patient samples with *PRMI* outer primers. Lane 2', patient 2 sample with *PRMI* nested primers. Lanes 3–9, patient samples with *PRMI* nested primers. Lane 10, one of the 39 ejaculates tested that do not express *PRMI* in a nested PCR assay. Lane 11, whole testis sample with outer and nested *PRMI* primers. *PRMI* PCR product was 150 bp; nested *PRMI* PCR product was 128 bp. M, f *Hae* III marker.

Even though a greater number of cells can be retrieved by using enzymatic digestion (Crabbé *et al.*, 1997), we have favoured the mechanical dissociation of the testicular biopsy in order to avoid a possible alteration of the cell membrane. As previously described for the mouse (Lassalle *et al.*, 1999), the centrifugation of the cell suspension obtained on a discontinuous Percoll gradient prior to flow cytometry resulted in a significant enrichment in round spermatids. In agreement with results in rat (Meistrich *et al.*, 1981) and in mouse (Lassalle *et al.*, 1999), most of the human round spermatids were recovered in the 22% Percoll fraction, while other workers attested their presence in the 70% Percoll fraction (Angelopoulos *et al.*, 1997; Vanderzwalmen *et al.*, 1997; Kahraman *et al.*, 1998). In our own experience, and according to Kolb *et al.* (1993), lymphocytes prepared from blood sedimented in that 70% Percoll gradient. The testis 22% Percoll fraction could be resolved in a very homogeneous population, showing all the characteristics assigned to round spermatids: a round shape, a round nucleus with a clearly visible nucleolus and surrounded by a continuous zone of cytoplasm and an acrosomal structure. Indeed, the sorted round spermatids clearly showed the characteristic cape covering half the nucleus surface, surmounted by the acrosomal granule which is the typical feature of a round spermatid in an advanced stage of development (i.e. step 5 to 7 of the cap phase, just before its elongating process starts) as initially described by Clermond and Leblond (1955). The diameter of the round spermatids obtained via the flow cytometry procedure is  $10.6 \pm 0.5 \mu\text{m}$ . It is close to the observations of Dadoune (1996), Fishel *et al.* (1997) Kahraman *et al.* (1998) and Verheyen *et al.* (1998), but it differs from the descriptions of Angelopoulos *et al.* (1997), Vanderzwalmen *et al.* (1997) and Tesarik *et al.* (1998) who attributed to round spermatids an average size of 5–8  $\mu\text{m}$ . It is noteworthy that Mendoza and Tesarik (1996) ascribed to the cells they have isolated the features of round spermatids in an early stage of development, i.e. the Golgi phase of spermiogenesis.

When subjected to flow cytometry, the lymphocyte population (average size,  $7.1 \pm 0.7 \mu\text{m}$ ) was sorted in a well-defined area, which cannot be confused with that of spermatids. In cases of activation (inflammatory process, lymphoma), the size of lymphocytes could be slightly increased. Nonetheless, the

size of the lymphoid cells we have observed in the ejaculate of non-obstructive azoospermic patients was similar to that of circulating blood lymphocytes.

The discrepancies concerning the size of the round spermatids coupled with the remaining uncertainty affecting the recognition of round spermatids admitted by various authors (Yamanaka *et al.*, 1997; Silber *et al.*, 1998; Vanderzwalmen *et al.*, 1998) enabled us to establish, without doubt, the identity of the cells we have sorted, using uncontroversial parameters such as the presence of mRNA corresponding to genes whose expression is restricted to the haploid germ cells, i.e. *PRMI* and *PRM2*. Such a proof would not be susceptible to the intra- and extra-observer variations resulting from visual identification under a microscope. The results of RT–PCR experiments clearly demonstrate that the sorted cell population expressed both *PRMI* and *PRM2*.

It was also of great importance to demonstrate that the population of round spermatids sorted was not heavily contaminated with other cell species. At the time ROSI is to be performed, the round spermatid chosen for microinjection can only be identified by eye. It is therefore essential that the source of spermatids should be as free of contaminating cells as possible, so that there will be no risk of selecting a wrong cell. To evaluate a possible contamination with either lymphocytes or diploid germ cells, we performed nested RT–PCR with primers specific to the two following genes: *CD3- $\delta$* , which codes for an antigen expressed ubiquitously at the surface of lymphoid cells (Van Den Elsen *et al.*, 1986); *MAGE1*, which codes for a tumour rejection antigen expressed in various cancers and also in spermatogonia and primary spermatocytes, with the exception of haploid germ cells (Takahashi *et al.*, 1995). Taking advantage of the extreme sensitivity of the nested one-step RT–PCR technique which can reveal the expression of a gene in a single cell, we proved that the round spermatid populations obtained via the flow cytometry procedure was free of either lymphocytes, spermatogonia or spermatocytes.

Once the standard parameters to isolate homogenous populations of spermatids from testicular biopsy cell suspensions has been established, we applied the procedure to the isolation of round spermatids from azoospermic patient ejaculates. Actually, the presence of spermatids in the ejaculates of these

patients is somewhat controversial. Mendoza and Tesarik (1996) attested their presence in 69% of the 124 observed azoospermic patients, whereas, after an extensive examination of the ejaculate of 36 patients, Antinori *et al.* (1997a) concluded that there was a complete absence of spermatogenic cells. Nevertheless, it is tempting to consider ejaculates as a favourable source of spermatids as this would overcome the risks associated with extensive sampling of testicular tissue in search of germ cells. Unfortunately, cell sorting coupled to flow cytometry did not permit the isolation of a spermatid population in the 14 ejaculates from the non-obstructive azoospermic patients analysed. In addition, among the 39 crude ejaculates analysed by RT-PCR, only nine were positive with the primers for *PRM1*. This means that only 23% of the non-obstructive azoospermic patients recruited in our ICSI centre exhibited some spermatids in their ejaculates and that these spermatids were too few to be isolated via the flow cytometry procedure.

Furthermore, these spermatids did not express protamine 2. These results are to be brought together with those of Yebra *et al.* (1998), who showed a marked reduction of the *PRM2* protein content in sperm cells of infertile patients. This poses the question of spermatid quality in ROSI programmes. Is this failure in expressing *PRM2* exceptional or widely distributed among non-obstructive azoospermic patients? Does it affect only ejaculated spermatids? Could it account in part for the very low fertilizing efficiency of spermatids? Are there other genes that are not normally expressed in the haploid germ cells of these patients?

To sum up, flow cytometry coupled to cell sorting seems to be the more powerful technique to provide homogeneous populations of spermatids from biopsies of men with obstructive azoospermia. The procedure offers decisive advantages, as it is fast, reliable, requires little tissue, as compared with elution which is time consuming and needs much more starting material.

### Acknowledgements

Ahmed Ziyyat was supported by a fellowship from the American Hospital of Paris (Neuilly sur Seine, 92202, France) and by a grant from the Ministère de l'Éducation Nationale et de la Recherche Scientifique (ACC SV4).

### References

- Angelopoulos, T., Krey, L., Mc Cullough, A. *et al.* (1997) A simple and objective approach to identifying human round spermatids. *Hum. Reprod.*, **12**, 2208–2216.
- Antinori, S., Versaci, C., Dani, G. *et al.* (1997a) Fertilization with human testicular spermatids: four successful pregnancies. *Hum. Reprod.*, **12**, 286–291.
- Antinori, S., Versaci, C., Dani, G. *et al.* (1997b) Successful fertilization and pregnancy after injection of frozen–thawed round spermatids into human oocytes. *Hum. Reprod.*, **12**, 554–556.
- Araki, Y., Motoyama, M., Yoshida, A. *et al.* (1997) Intracytoplasmic injection with late spermatids: a successful procedure in achieving childbirth for couples in which the male partner suffers from azoospermia due to deficient spermatogenesis. *Fertil. Steril.*, **67**, 559–561.
- Aslam, I., Robins, A., Dowell, K. *et al.* (1998) Isolation, purification and assessment of viability of spermatogenic cells from testicular biopsies of azoospermic men. *Hum. Reprod.*, **13**, 639–645.
- Chen, E.Y., Cheng, A., Lee, A. *et al.* (1991) Sequence of human glucose-6-phosphate dehydrogenase cloned in plasmids and a yeast artificial chromosome. *Genomics*, **10**, 792–800.
- Clermont, Y. and Leblond, C.P. (1955) Spermiogenesis of man, monkey, ram and other mammals as shown by the periodic acid–Schiff technique. *Am. J. Anat.*, **96**, 229–254.
- Crabbe, E., Verheyen, G., Tournaye, H. *et al.* (1997) The use of enzymatic procedures to recover testicular germ cells. *Hum. Reprod.*, **12**, 1682–1687.
- Dadoue, J. P. (1997) La spermatide, cette méconnue. *Andrologie*, **7**, 22–33.
- Devroey, P., Liu, J., Nagy, Z. *et al.* (1995) Pregnancies after testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) in non obstructive azoospermia. *Hum. Reprod.*, **10**, 1457–1460.
- Domenjoud, L., Nussbaum, G., Adham, I. *et al.* (1990) Genomic sequences of human protamines whose genes, *PRM1* and *PRM2*, are clustered. *Genomics*, **8**, 127–133.
- Edwards, R., Tarin, J., Dean, N. *et al.* (1994) Are spermatid injections into human oocytes now mandatory? *Hum. Reprod.*, **9**, 2217–2219.
- Fishel, S., Green, S., Bishop, M. *et al.* (1995) Pregnancy after intracytoplasmic injection of spermatid. *Lancet*, **245**, 1641–1642.
- Fishel, S., Green, S., Hunter, A. *et al.* (1997) Human fertilization with round and elongated spermatids. *Hum. Reprod.*, **12**, 336–340.
- Kahraman, S., Polat, G., Samli, M. *et al.* (1998) Multiple pregnancies obtained by testicular spermatid injection in combination with intracytoplasmic sperm injection. *Hum. Reprod.*, **13**, 104–110.
- Kimura, Y. and Yanagimachi, R. (1995) Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development*, **121**, 2397–2405.
- Kolb, J.P., Abadie, A., Paul-Eugène, N. *et al.* (1993) Ligation of CD23 triggers cyclic AMP generation in human B lymphocytes. *J. Immunol.*, **150**, 4798–4809.
- Lassalle, B., Ziyyat, A., Testart, J. *et al.* (1999) Flow cytometric method to isolate round spermatids from mouse testis. *Hum. Reprod.*, **14**, in press.
- Meistrich, M., Longtin, J., Brock, W. *et al.* (1998) Purification of rat spermatogenic cells and preliminary biochemical analysis of these cells. *Biol. Reprod.*, **25**, 1065–1077.
- Mendoza, C. and Tesarik, Y. (1996) The occurrence and identification of round spermatids in the ejaculate of men with nonobstructive azoospermia. *Fertil. Steril.*, **66**, 826–829.
- Ogura, A., Matsuda, J. and Yanagimachi, R. (1994) Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc. Natl. Acad. Sci. USA*, **91**, 7460–7462.
- Silber, S. and Johnson, L. (1998) Are spermatid injections of any clinical value? ROSNI and ROSI revisited. *Hum. Reprod.*, **13**, 509–515.
- Silber, S., Van Steirteghem, A.C., Nagy, Z. *et al.* (1996) Normal pregnancies resulting from testicular sperm extraction and intracytoplasmic sperm injection for azoospermia due to maturation arrest. *Fertil. Steril.*, **66**, 110–117.
- Sofikitis, N., Miyagawa, I., Agapitos, E. *et al.* (1994) Reproductive capacity of the nucleus of the male gamete after completion of meiosis. *J. Assist. Reprod. Genet.*, **11**, 325–341.
- Sofikitis, N., Toda, T., Miyagawa, I. *et al.* (1996) Beneficial effects of electrical stimulation before round spermatid nuclei injections into rabbit oocytes on fertilization and subsequent embryonic development. *Fertil. Steril.*, **65**, 176–185.
- Sofikitis, N., Yamamoto, Y., Miyagawa, I. *et al.* (1998) Ooplasmic injection of elongating spermatid for the treatment of non-obstructive azoospermia. *Hum. Reprod.*, **13**, 709–714.
- Takahashi, K., Shichijo, S., Noguchi, M. *et al.* (1995) Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. *Cancer Res.*, **16**, 3478–3482.
- Talbot, P. and Chacon, R.S. (1981) A triple-stain technique for evaluating normal acrosome reaction of human sperm. *J. Exp. Zool.*, **215**, 201–208.
- Tesarik, Y. and Mendoza, C. (1996) Spermatid injection into oocytes. I. Laboratory techniques and special features of zygote development. *Hum. Reprod.*, **11**, 772–779.
- Tesarik, J., Mendoza, C. and Testard, J. (1995) Viable embryos from injection of round spermatids into oocytes. *N. Engl. J. Med.*, **333**, 525.
- Tesarik, J., Rolet, F., Brami, C. *et al.* (1996) Spermatid injection into human oocytes. II. Clinical application in the treatment of infertility due to non-obstructive azoospermia. *Hum. Reprod.*, **11**, 780–783.
- Tesarik, Y., Greco, E. and Mendoza, C. (1998) ROSI, instructions for use: 1997 update. *Hum. Reprod.*, **13**, 519–523.
- Van den Elson, P., Georgopoulos, K., Shepley, B.A. *et al.* (1986) Exon/intron organization of the genes coding for the delta chains of the human and

- murine T-cell receptor/T3 complex. *Proc. Natl. Acad. Sci. USA*, **83**, 2944–2948.
- Van der Bruggen, P., Traversari, C., Chomez, P. *et al.* (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*, **254**, 1643–1647.
- Vanderzwalmen, P., Lejeune, B., Nijs, M. *et al.* (1995) Fertilization of an oocyte microinseminated with a spermatid in an in-vitro fertilization programme. *Hum. Reprod.*, **10**, 502–503.
- Vanderzwalmen, P., Zech, H., Birkenfeld, A. *et al.* (1997) Intracytoplasmic injection of spermatids retrieved from testicular tissue: influence of testicular pathology, type of selected spermatids and oocyte activation. *Hum. Reprod.*, **12**, 1203–1213.
- Vanderzwalmen, P., Nijs, M., Schoysman, R. *et al.* (1998) The problems of spermatid microinjection in the human: the need for an accurate morphological approach and selective methods for viable and normal cells. *Hum. Reprod.*, **13**, 515–519.
- Verheyen, G., Crabbé, E., Joris, H. *et al.* (1998) Simple and reliable identification of the human round spermatid by inverted phase contrast microscopy. *Hum. Reprod.*, **13**, 1570–1577.
- Yamanaka, K., Sofikitis, N., Miyagawa, I. *et al.* (1997) Ooplasmic round spermatid nuclear injection procedures as an experimental treatment for nonobstructive azoospermia. *J. Assist. Reprod. Genet.*, **14**, 55–62.
- Yebra de, L., Balleca, J.L., Vanrell, J. *et al.* (1998) Detection of P2 precursors in the sperm cells of infertile patients who have reduced protamine P2 levels. *Fertil. Steril.*, **69**, 755–759.

Received on July 31, 1998; accepted on October 29, 1998