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The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients

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BACKGROUND: Before clinical application, the feasibility and safety of autologous testicular stem cell transplantation should be explored. Apart from limitations in their numbers, spermatogonial stem cells may also be contaminated by malignant cells. Therefore, both enrichment and decontamination before transplantation may be necessary. This study aimed at evaluating the decontaminating potential of magnetic-activated cell sorting (MACS) and/or fluorescence-activated cell sorting (FACS) for both murine and human testicular cell suspensions. In the mouse, the effectiveness of the transplantation technique after cell sorting was also assessed. METHODS: Murine testicular cells were contaminated with 5% EL4 cells. Fresh and frozen–thawed suspensions were sorted using MACS (CD49f$^+$) and FACS (CD49f$^+$, H-2Kb$^-$) and evaluated by FACS, cell culture and transplantation into W/W^v mice. Human testicular cells were contaminated with 5 or 0.05% CCRF-SB (SB) cells. Frozen–thawed suspensions were sorted using FACS (HLA class I$^-$) and evaluated by FACS, cell culture and PCR for the B-cell receptor. RESULTS: In the mouse, the sorted fractions contained 0.39% H-2K^b-positive and 76.55% CD49f-positive cells. After transplantation, 1 in 20 recipient mice developed a malignancy. In the human experiments, an average of 0.58% SB cells was detected after sorting. In only 1 of 11 samples, there were no SB cells observed. CONCLUSION: MACS and/or FACS are insufficient for completely depleting testicular tissue of malignant cells. Although more research on alternative decontamination techniques is necessary, developing a reliable method to screen *a priori* testicular tissue for malignant cells may be equally important.

Key words: spermatogonia/decontamination/transplantation/cancer

Introduction

The storage of prepubertal testicular tissue before autologous transplantation of testicular stem cells is emerging as a potential solution for fertility preservation in prepubertal cancer patients (for review see Tournaye *et al*., 2004). The transplantation technique has already proven successful in animal models (Brinster and Zimmerman, 1994; Ogawa *et al*., 1999; Honaramooz *et al*., 2002), including primates (Schlatt *et al*., 2002). However, before applying this procedure in clinical practice, the feasibility and safety of this technique—apart from ethical considerations (for review see Bahadur, 2004 and Bahadur *et al*., 2000)—should be studied.

On the one hand, in a clinical setting, one small prepubertal testis at the most can be removed for cryopreservation and future autologous testicular stem cell transplantation. Because spermatogonial stem cells represent only a small percentage of the testicular cells [estimated 0.03% in mouse; in human this percentage is assumed to be higher (Meistrich and van Beek, 1993)] and because the effectiveness of the transplantation technique is related to the number of stem cells transplanted (Dobrinski *et al*., 1999), enrichment of stem cells in the suspensions for transplantation may be necessary. On the other hand, in case of leukaemia or any other metastatic childhood cancer, there is a risk of contamination of the harvested testicular cells with carcinogenic cells. In this case, testicular stem cell transplantation may induce a malignant relapse in the transplanted patient. Jahnukainen *et al*. (2001) demonstrated in a rat model that transplantation of as few as 20 leukaemic cells could cause malignant recurrence in the recipient animal. Therefore, apart from enrichment, decontamination of the cell suspensions before transplantation may also be necessary in patients at risk.

Cell sorting by magnetic-activated cell sorting (MACS) and/or fluorescence-activated cell sorting (FACS) may be a solution to obtain both enrichment and decontamination. This study, therefore, aimed at evaluating the decontamination potential of these two cell sorting techniques for both murine

and human testicular cell suspensions contaminated with carcinogenic cells. The effectiveness of the transplantation technique with murine testicular cell suspensions after cell sorting by MACS and FACS was also assessed.

Materials and methods

Murine experiments

The study design for the murine experiments is shown in Figure 1.

Preparation of the cells

Donor cells were obtained from B6CBAF$_1$/Juco mice (Iffa Credo, France) that were made cryptorchid at the age of 6 weeks, that is, 2 months before the collection of the testicular cells. The testes were decapsulated, and the tubuli seminiferi were transferred for 1 h in a 37°C water bath in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM), supplemented with F12 nutrient (HEPES-DMEM/F12, Invitrogen, Belgium) containing 2 mg/ml collagenase IV (C-5138; Sigma, Belgium). After washing, the cells were resuspended in a trypsine solution, containing 0.25 mg/ml trypsin (T-4665; Sigma) and 0.01 mg/ml desoxyribonuclease (DNase, DN25, Sigma) in HEPES-DMEM/F12, incubated for 15 min in a 37°C water bath, then washed and resuspended in HEPES-DMEM/F12.

The tumour cells used in the murine experiments were EL-4 cells (ATCC, UK), which are T-lymphoma cells, raised in C57Bl/6N mice by treatment with the 9,10-dimethyl-1,2-benzathracene mutagen (Gorer, 1950). The EL-4 cells were kept in culture in HEPES-DMEM/F12, supplemented with 2% fetal bovine serum [(FBS) 10500–056; Invitrogen] and 1% penicillin–streptomycin [(pen/strep) 15070–063; Invitrogen].

Before labelling of the cells, the testicular cell suspensions were contaminated with 5% EL-4 cells. Part of the suspensions were frozen–thawed; the rest was kept fresh. For freezing, cryopreservation medium was added to the cell suspension to a final concentration of cryoprotectants of 0.15 M sucrose (10274; BDH Laboratory Supplies, UK) and 1.5 M ethylene glycol (E-9129; Sigma) with 10% FBS and 0.01 g/ml bovine serum albumin [(BSA) A-9647; Sigma]. The suspensions were loaded into straws (0.5 ml, L5780; l'Air Liquide, Belgium) with two drops of HEPES-DMEM/F12 at the beginning and end of the straw to prevent contact of the cell suspension with water during thawing. The straws were transferred into a biofreezer (Embryo Freeze; Biotronics Ltd, UK) at a starting temperature of 5°C. The temperature was decreased by 1°C/min down to –10°C. At the seeding temperature of –7°C, there was a 10-min pause in the programme. The temperature was then decreased by 3°C/min down to –20°C and by 5°C/min down to –80°C (Izadyar *et al*., 2002; Woelders and Chaveiro, 2004). At this point, the straws were quickly transferred into liquid nitrogen where they were preserved for at least 24 h. For thawing of the cell suspensions, the straws were put in a 37°C water bath for 15 s. The cells were then washed and resuspended in phosphate-buffered salt solution [(PBS) P 4417; Sigma)].

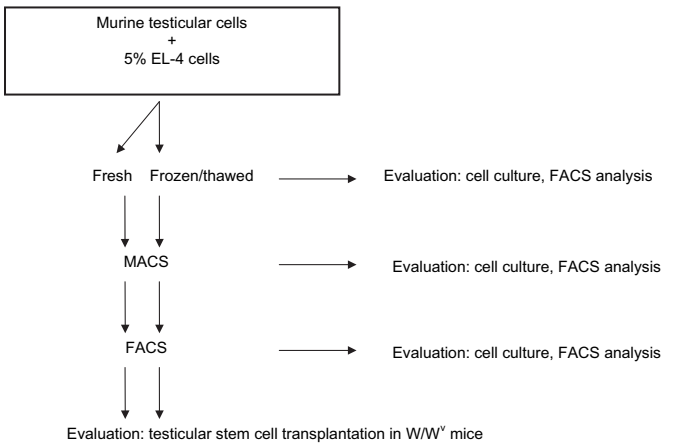

Figure 1. Setup of the murine decontamination experiments. Testicular cells were contaminated with 5% EL-4 tumour cells. Fresh and frozen–thawed suspensions were sorted using magnetic-activated cell sorting (MACS) (CD49f$^+$) and fluorescence-activated cell sorting (FACS) (CD49f$^+$, H-2K^{b-}). After each step, the suspensions were evaluated by FACS analysis and cell culture, and the final suspension was transplanted into W/W^v mice.

Cell sorting

The dissociated cells were incubated with a R-Phycoerythrin (R-PE)-conjugated rat anti-CD49f monoclonal antibody (555736; PharMingen, Belgium) at 20 µl per 10^6 cells for 20 min, at 4°C, in the dark and washed three times with excess PBS. CD49f or α_6-integrin is a part of the laminin receptor that is expressed by spermatogonial stem cells (Shinohara and Brinster, 2000; Shinohara *et al*., 1999, 2000). In a second step, the cells were identically treated with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-H-2K^b monoclonal antibody (553569; PharMingen) at 2 µl per 10^6 cells. H-2K^b is a part of the murine major histocompatibility complex (MHC) class I molecule, which is not expressed on spermatogenic cells (Kubota *et al*., 2003). In a final labelling step, 80 µl MACS buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) per 10^7 cells and 20 µl anti-PE MACS Microbeads® (130–048–801; Miltenyi Biotec, Belgium) per 10^7 cells were added to the cell pellet. After mixing, the cells were incubated in the dark for 15 min at 4°C. The cells were washed with excess MACS buffer and resuspended in 500 µl buffer per 10^7 cells.

As a first strategy for enriching the suspensions with CD49f-positive cells, MACS was performed. The magnetic separation of the cells was performed using a LS-column (Miltenyi Biotec) that was placed in a VarioMACS separator (Miltenyi Biotec), as described by the manufacturer's protocol. The positive selected cells were finally suspended in 1 ml MACS buffer per 4×10^6 total cells.

FACS was performed with a FACS Vantage Flow Cytometer (BD Biosciences, Belgium). The 488-nm argon laser was used at 50 mW to excite FITC and PE, and fluorescence emissions were collected with a 530/30-nm filter for FITC and a 585/42-nm filter for PE. The goal of the murine experiment was not only to decontaminate the cell suspensions but also to enrich the sorted fractions for CD49f-positive cells, thereby optimizing the conditions for the testicular stem cell transplantation. Two parameters were used to select the cells: the positively sorted cells had to be PE-positive and FITC-negative, which means that they had to express the CD49f protein but not H-2K^b. The validity of these markers was checked on PE/FITC parameters with a pure population of EL-4 cells (see Figure 2A). Gate 1 (R1), as selection for the tumour cells, and gate 2 (R2), as selection for the stem cell-enriched fraction, were set on PE/FITC parameters (see Figure 2B). The sorting of the cells was performed using the parameters 'R2 and not R1'. The sorted cells were collected into polypropylene 5-ml tubes (352063; Falcon, VWR) containing 1.5 ml DMEM supplemented with 10% FBS and 10% pen/strep. Data for at least 20 000 events were collected with the software program Cell Quest version 3.3 (BD Biosciences). To exclude non-specific binding of the antibodies, samples of the suspensions were also labelled with an isotype control. This was a FITC-conjugated mouse-IgG2a (349051; PharMingen) for anti-H-2K^b and a PE-conjugated rat-IgG2a (555844; PharMingen) for anti-CD49f. The FACS analysis results were corrected for these isotype controls.

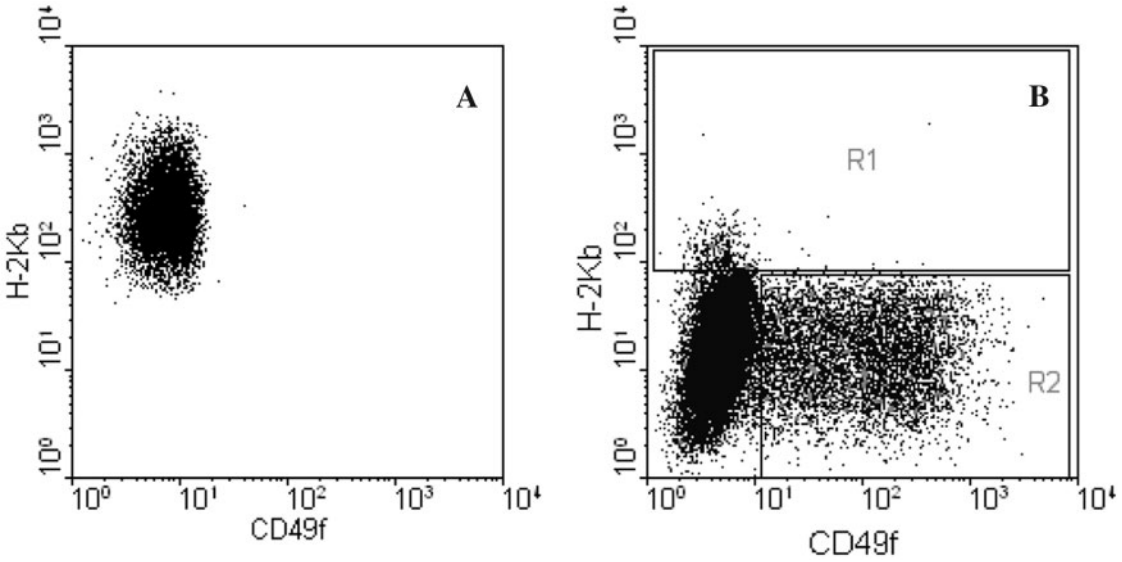

Figure 2. (**A**) Fluorescence-activated cell sorting (FACS) analysis of a pure population of EL-4 cells, labelled for H-2K^b and CD49f. All cells were H-2K^{b+}/CD49f$^-$. (**B**) Sorting parameters for the murine decontamination experiments: gate 1 (R1), as selection for the tumour cells (H-2K^{b+} cells), and gate 2 (R2), as selection for the stem cell-enriched fraction (CD49f$^+$ cells), were set on phycoerythrin/fluorescein isothiocyanate (PE/FITC) parameters. Cell sorting was performed using the parameters 'R2 and not R1'.

Evaluation

At every step in the sorting experiment, samples of the cells were analysed using the FACS Vantage Flow Cytometer to determine positivity for CD49f and H-2K^b. The software program Win MDI, version 2.8, was used to analyse the data.

Next to FACS analysis, 10 000 cells/well were put in culture in a 4-well plate, containing 1 ml of culture medium (HEPES-DMEM/F12 supplemented with 2% FBS and 1% pen/strep) in each well and evaluated for tumour cell colonies. The medium was changed every 3 days, and the cultures were maintained for 18 days. To evaluate the sensitivity of this evaluation technique, a dilution series of tumour cells in 10 000 testicular cells was set up in the same culture conditions.

In vivo evaluation of decontamination was obtained using the final sorted cells for testicular stem cell transplantation. Four- to six-week old, sterile male W/W^v mice (Jackson Labs, USA) were used as recipients for the testicular stem cell transplantation. The transplantation was performed under a stereomicroscope as described before (Goossens *et al*., 2003). The transplantation pipette was introduced into the rete testis via the efferent duct. The transplantation procedure was considered successful when a volume of 2.5–5 µl, equivalent to 5–10 $\times 10^3$ cells, was injected into the tubules. Immediately after transplantation, the mice were injected s.c. with 100 µl of antibiotics [0.125% Baytril (Bayer, Belgium) in saline]. The recipient mice were evaluated after their natural death or at least 120 days after transplantation. Their abdomen was evaluated for the presence of tumours, and the testes and possible tumours were isolated and prepared for histological evaluation as previously described (Geens *et al*., 2006). The collected tissue was fixed in Bouin's fixative and afterwards dehydrated by treatment with Vacuum Infiltration Processor (Bayer, Germany) and embedded in paraffin. Cross-sections of 5 µm thickness were examined by light microscopy after haematoxylin–eosin staining at an ×200 magnification. An adult W/W^v male mouse was used as a negative control. To evaluate the sensitivity of this evaluation technique, 44 W/W^v mice were transplanted with different numbers of EL-4 cells, and tumour formation was evaluated after natural death or 120 days after transplantation.

Viability of the cells

To examine the viability of the cells during the cell sorting procedure, three experiments were conducted under the same conditions as the decontamination experiments. After digestion of the testicular tissue, labelling of the cells with the antibodies and both MACS and FACS, percentages of viable and dead cells were determined. Calcein AM, 0.5 µl, and 0.5-µl ethidium homodimer (L-3224, Invitrogen) were added to 10 µl of a cell suspension. The samples were examined under a fluorescence microscope (BH2-RFCA; Olympus) with UV light and BP filter 405. Three hundred cells were counted for each sample. Viable cells are characterized by esterase activity, converting calcein AM to the green fluorescent calcein. Ethidium penetrates passively into dead cells, where it binds as a red fluorescent molecule to the DNA.

Human experiments

The study design for the human experiments is shown in Figure 3.

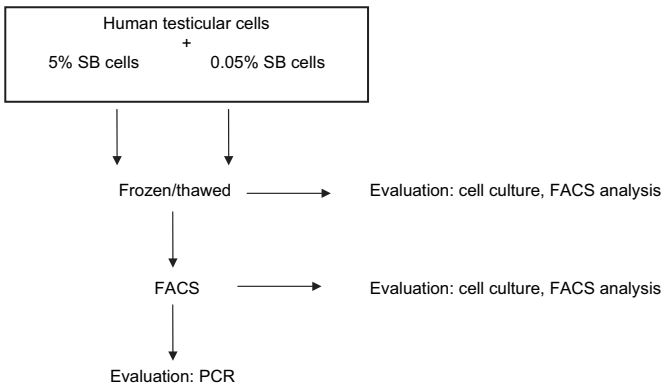

Figure 3. Setup of the human decontamination experiments. Testicular cells were contaminated with 5 or 0.05% SB cells. Frozen–thawed suspensions were sorted using fluorescence-activated cell sorting (FACS) (HLA class I$^-$). The sorted suspensions were evaluated by FACS analysis, cell culture and PCR for the B-cell receptor.

Preparation of the cells

The human testicular tissue was obtained, after written informed consent, from patients undergoing vasectomy reversal. All men had normal spermatogenesis as proven by histology. The tissue was digested according to a modified technique described by Crabbe *et al.*, 1997, 1998). Per 20–30 mg of testis tissue, 1 ml of digestion medium was added to the tube. This medium contained 1000 IU/ml collagenase IV-S (C1889; Sigma) and 0.125 mg/ml calcium chloride ($CaCl_2$, C-7902; Sigma) in HEPES-DMEM/F12 with 1% human serum albumin [(HAS) CAF Red Cross, Belgium]. The cells were incubated for 1 h in a shaking 37°C water bath, and the tube was vortexed every 10 min. After incubation, excess of HEPES-DMEM/F12 supplemented with 1% HSA was added to dilute the enzymes. The suspensions were washed three times with PBS supplemented with 0.1% BSA.

The tumour cells used in the human experiments were SB cells (ATCC). These cells were obtained from a patient with a B-cell acute lymphoblastic leukaemia (B-ALL) which is the most prevalent leukaemic cell type in children (Sinnett *et al.*, 2006). The culture conditions were identical with those of the murine EL-4 cells.

The testicular cells were contaminated with 5% (for 5 patients) and 0.05% (for 6 patients) SB cells before freezing and thawing using the same protocols as in the murine setup.

Cell sorting

A FITC-conjugated mouse anti-HLA class I monoclonal antibody (555552; PharMingen) was added to the cell suspensions at 8 µl per 10^6 cells, and the cells were incubated in the dark for 20 min at 4°C. SB cells, like most cells, express HLA class I molecules, whereas spermatogenic cells do not. After labelling, the cells were washed three times with excess PBS supplemented with 0.1% BSA.

Because, compared with murine tissue, the quantity of patient material is limited, no prior MACS was performed. The main goal of the human experiments was to decontaminate the testicular cell suspensions. Gate 1 (R1) was therefore set on PE/FITC parameters (see Figure 4B) and contained all HLA class I (FITC) positive cells, and only one selection parameter was used, 'not R1'. The validity of this marker was checked with a pure population of SB cells (see Figure 4A). The sorted cells were collected into polypropylene 5-ml tubes (352063; Falcon, VWR, Belgium) containing 1.5 ml DMEM supplemented with 2% FBS and 1% pen/strep. Data for at least 20 000 events were collected with the software program Cell Quest version 3.3 (BD Biosciences). As isotype control for the anti-HLA class I antibody, a FITC-conjugated mouse IgG1 (555748; PharMingen) was used.

Evaluation

As in the murine experiments, samples of the cells were analysed using the FACS Vantage Flow Cytometer. Positivity for HLA class I was determined using the software program Win MDI, version 2.8.

In parallel with FACS analysis, 10 000 cells/well were put in culture in a 4-well plate, containing 1 ml of culture medium in each well, and evaluated for tumour cell colonies, according to the same protocols as in the murine experiments. Also, a similar sensitivity trial was conducted with the SB cells as with the EL-4 cells.

Paulus *et al.* (1997) described that suspensions that were negative for tumour cells, as assessed by FACS after tumour cell depletion, could still be positive when evaluated by PCR. Therefore, we used a PCR for the B-cell receptor as a more sensitive test to evaluate SB cell presence. After sorting the cells, DNA was extracted from the positively sorted fraction. This extraction was performed with a QIAamp® DNA Blood Mini Kit (51106; Qiagen, Belgium), according to the protocol of the producer. Up to 5×10^6 cells, suspended in 200 µl PBS, were lysed by adding 20 µl of QIAGEN Protease and 200 µl of Buffer AL. This mix was vortexed and incubated for 10 min at 56°C. Then, 200 µl of ethanol was added, and the sample was applied to a QIAamp Spin Column. The column was centrifuged at 4.3×10^3 *g* for 1 min, and the flow through was discarded. Two washing steps were performed with 500 µl of Buffer AW1 and 500 µl of Buffer AW2. An extra centrifugation was performed at 1.2×10^4 *g* for 1 min to eliminate possible residual buffer. Five elutions were performed by adding 50 µl of milliQ water to the column, incubating for 5 min at room temperature and centrifugating for 1 min at 4.3×10^3 *g*.

The PCR was performed with 500 ng DNA in a 50 µl final reaction volume comprising 10% reaction buffer (Roche Diagnostics, Belgium),

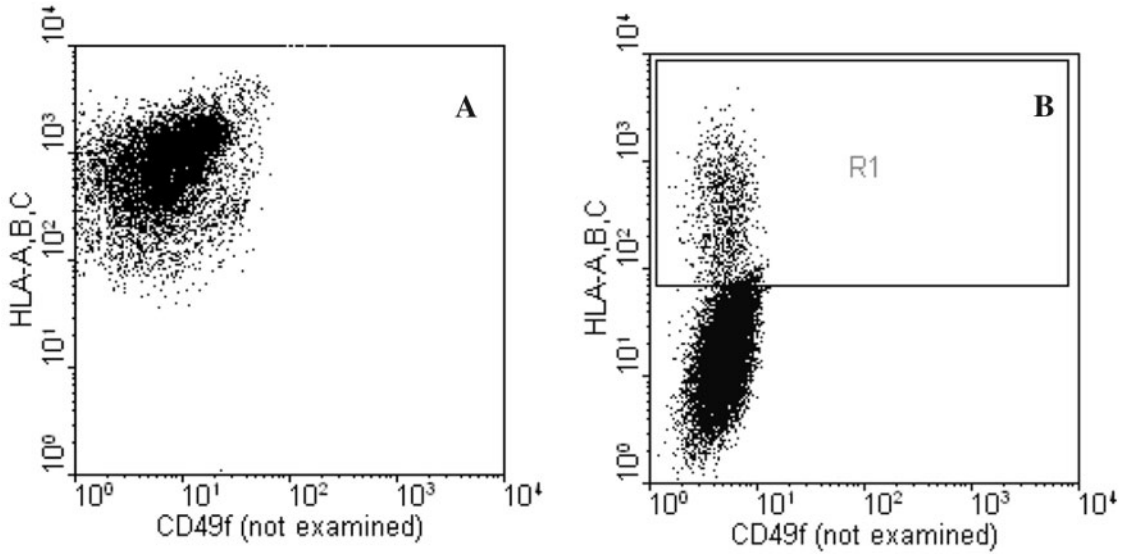

Figure 4. (**A**) Fluorescence-activated cell sorting (FACS) analysis of a pure population of SB cells, labelled for HLA class I. All cells were positive for this marker. (**B**) Sorting parameters for the human decontamination experiment: gate 1 (R1) was set on phycoerythrin/fluorescein isothiocyanate (PE/FITC) parameters and contained all HLA class I (fluorescein isothiocyanate [FITC]) positive cells. Only one selection parameter was used, 'not R1'.

2.2 mM dNTP (Amersham Pharmacia Biotech, Belgium), 1.4 IU Expand High Fidelity Taq polymerase (Roche Diagnostics) and with 5 μM of a specific primer mix, consisting of the forward primer (5′-ACACGGCYSTGTATTACTG-3′) labelled with 5′-indocarbocyanine (Cy5) and the reverse primer (5′-CTTACCTGAGGAGACGGTGACC-3′) (Eurogentec, Belgium), coding for a 120-bp fragment of the B-cell receptor. A standard single-cell PCR protocol was applied [5 min 95°C, 50 cycles (30 s 95°C, 30 s 52°C, 30 s 72°C), 7 min 72°C] in an Eppendorf Master Cycler (VWR International, Belgium). Fluorescent fragments were analysed on an Automated Laser Fluorescence Express DNA sequencer (ALFExpress, Amersham Pharmacia Biotech). In parallel with the patients' DNA, three negative controls (H_2O, DNA derived from EL-4 cells and DNA derived from non-contaminated testicular biopsies) were put into the reaction mix.

Statistics

To check for differences between the fresh and frozen–thawed samples in the murine experiments, and between the samples contaminated with 5% or with 0.05% SB cells in the human experiments, the change scores of the FACS results were compared using a *t*-test. *P*-values below 0.05 were considered significant.

Internal review board approval

All experiments in this study were approved by both the Ethical Review Board of the University Hospital and the Animal Care and Use Committee of the Brussels Free University.

Results

Murine experiments

FACS analysis

The goal of the murine experiment was both to decontaminate the testicular cell suspensions by eliminating all EL-4 cells (H-2K^{b+}) and to enrich the suspensions with spermatogonial stem cells (CD49f^{+}). These markers proved to be valid to clearly differentiating between germ cells and EL-4 cells. In Figure 2A, it is shown that all EL-4 cells expressed H-2K^{b}, whereas none of them were positive for CD49f. A clear enrichment of CD49f^{+} cells was observed: from an average of only 3.94% at the start of the experiments, their proportion increased to 40.46% after MACS and eventually 76.55% after additional FACS (see Table I). On the contrary, H-2K^{b}-positive cells were removed from the suspensions: at the start of the experiment, 10.35% of the cells in the suspension expressed this marker. After magnetic separation, their proportion decreased to 3.54% and after FACS, only 0.39% of H-2K^{b+} cells was observed (Table I). No significant difference was observed when comparing the fresh and frozen–thawed samples.

Cell culture

In the preliminary sensitivity test, only from a dilution of 16 EL-4 cells in 10 000 murine testicular cells onwards, tumour cell colonies were observed in all wells (10 out of 10; see Figure 5). Before sorting, tumour growth was detected in all the wells. After MACS, tumour cell colonies could still be found in 50% of the wells, but after additional FACS, only 1 of 32 wells (3.1%) was positive.

Transplantation

In the tumour load experiment that was conducted to test the sensitivity of this evaluation technique, we observed tumour formation in 43% of the mice injected with 5–39 EL-4 cells. This percentage was higher in the groups with 40–100 and with more than 10 000 tumour cells but never reached 100% (see Table II). Eleven W/W^{v} mice were transplanted with fresh suspensions, nine with frozen–thawed suspensions. The transplantation procedure was successful in 11 testes transplanted with fresh suspensions and in 9 testes transplanted with frozen–thawed suspensions. After evaluation of the testes transplanted with fresh-sorted fractions, spermatogenesis was observed in four of the testes (36.4%). In none of the testes transplanted with frozen–thawed sorted suspensions was spermatogenesis or colonization of spermatogonial stem cells observed. In one of the 20 mice that were transplanted in this experiment, an abdominal tumour was observed (see Figure 6). This mouse was transplanted with 8000 fresh cells diffusely in the interstitium of the right testicle and 6000 fresh cells into the rete testis of the left testicle. On the right, the testis was recovered, without any sign of spermatogenesis. On the left, two pieces of a solid tumour were found, but no remaining testis was observed.

Cell viability

Immediately after digestion of testicular tissue, the viability of the cells was 70.5% ± 0.6%. This percentage was retained after labelling (69.7% ± 0.6%) and MACS (69.1% ± 1.6%). After

Table I. FACS analysis of the murine testicular cell suspensions

Murine experiment	H-2K^{b}-positivity (%) (Corrected for isotype control)			CD49f positivity (%) (Corrected for isotype control)		
	Before sort	After MACS	After FACS	Before sort	After MACS	After FACS
Fresh suspensions						
1	6.82	4.46	0.68	3.08	45.45	92.65
2	12.61	7.42	0.67	5.76	61.07	68.10
3	17.25	3.21	0.51	8.35	63.76	92.98
4	18.92	5.13	0.64	4.31	49.70	76.39
5	9.15	5.77	0.00	4.27	22.93	66.75
6	5.23	0.67	0.49	2.36	36.31	54.71
Frozen–thawed suspensions						
7	9.38	1.22	0.14	1.45	18.76	84.01
8	3.46	0.40	0.00	1.92	25.68	76.78
Average (SD)	10.35 (± 5.54)	3.54 (± 2.59)	0.39 (± 0.30)	3.94 (±2.28)	40.46 (±17.29)	76.55 (±13.25)

FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting.

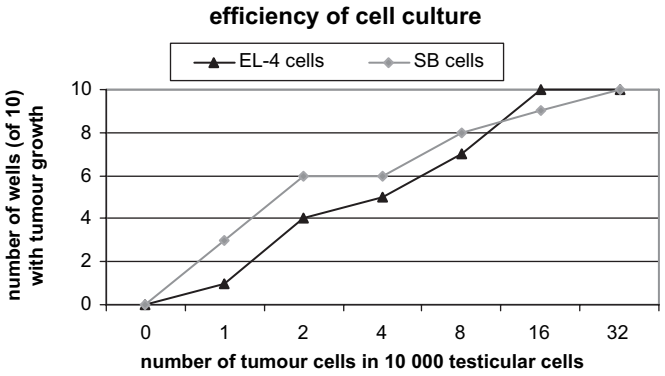

Figure 5. Graphical overview of the results of the sensitivity test of *in vitro* cell culture to evaluate cancer cell contamination. A dilution series of tumour cells (EL-4 for murine experiments, SB for human experiments) in 10 000 testicular cells was evaluated for tumour cell colonies. For each dilution, 10 culture wells were evaluated.

additional FACS, still 60.5% ± 0.8% of the cells was positive for calcein; however, a lot of cell debris was observed within these samples.

Human experiments

Sensitivity of the evaluation techniques

Because all SB cells expressed HLA class I, as shown in Figure 4A, this marker could be successfully used to differentiate between SB cells and germ cells.

To evaluate the sensitivity of our cell culture, a dilution series of SB cells in 10 000 human testicular cells was set up. Only at the highest concentration of tumour cells, that is, 32 SB cells per well, were tumour cell colonies observed in all of the 10 wells (see Figure 5).

Our PCR protocol for the B-cell receptor was able to detect 50 pg of SB cell DNA in a total amount of 500 ng DNA. This means that a dilution sensitivity of 1/10 000 could be reached or that it should be possible to detect 1 SB cell per 10 000 testicular cells. The high number of reaction cycles did not lead to false positive results, as could be proven by testing different samples of non-contaminated human testicular cells (data not shown because experiments were conducted in preliminary tests) and samples of murine EL-4 cells (see Figure 7), all of which produced a negative result.

Patient samples

The sorted suspensions of 10 out of 11 patients that were evaluated in these experiments still contained SB cells (0.58%), as proven by FACS analysis, cell culture and PCR (see Tables III and IV and Figure 7). Only for patient 5b, whose testicular cell suspension was contaminated with 0.05% tumour cells, were no SB cells detected after sorting (see Table IV). No significant differences were observed between the two groups of patients, that is 5 or 0.05% contamination.

Discussion

At present, prepubertal cancer patients have no options to preserve their fertility after a sterilizing cancer therapy. Testicular stem cell transplantation may become a promising technique, but the possible contamination of the testicular tissue with malignant cells is a potential barrier for a safe clinical application. Recently, Fujita *et al*. (2005) reported restoration of fertility in sterile mice by transplanting spermatogonial stem cells, isolated from leukaemic mice, without inducing leukaemia in the recipients. Although our results seem to contradict this promising report, the authors did not re-analyse the sorted fractions by any other method than by *in vivo* transplantation in 6 recipient mice and therefore could not rule out the presence of a few leukaemic cells in the germ cell-enriched fraction they had transplanted. We were able to demonstrate that in a murine setup ~0.4% of H-2K^{b}-positive cells were still present in the sorted fraction after MACS (CD49f^{+}) and FACS (CD49f^{+}, H-2K^{b-}) and that these cells could develop colonies *in vitro* and tumours *in vivo*. Also, in the human setup, we demonstrated by cell culture and PCR that FACS purification is not sufficiently efficient to ensure a total depletion of malignant cells from a testicular cell suspension. Only in 1 of 11 suspensions, could no B cells be detected with a detection level of 1 per 10 000 cells.

In a clinical setup, the transfer of as few as one single cell should theoretically be avoided. Sorting the cells by FACS will not be sufficient for achieving a complete depletion of malignant cells. In model studies, bone marrow with 10% tumour cell contamination could be purged by a factor of 2–4 logs using FACS, meaning that 0.1% of malignant cells could still be returned to the patient (Anderson *et al*., 1989; Gross *et al*., 1995). Major problems for the efficiency of this cell sorting technique are the low detection level for cancer cells (at best 1 even among 10^4–10^5 sorted cells), possible phenotypical variation of surface marker expression by cancer cells and possible aggregation of cancer cells with germ cells (Jahnukainen *et al*., 2006).

It appears from our results that more research on alternative decontamination techniques is necessary. One of the techniques currently being studied for fertility preservation is the xenografting of testicular tissue to an immunodeficient recipient (Geens *et al*., 2006; Schlatt *et al*., 2006). Apart from being a potential solution for fertility preservation, this technique could be used to avoid contamination by malignant cells by using the developed spermatozoa for ICSI. Alternatively, this

Table II. Tumour load experiment

Number of EL-4 cells injected	<5	5–39	40–100	>10 000	Control	Total
Mice with tumour	0 (0.0%)	6 (42.9%)	4 (80.0%)	6 (75.0%)	0 (0.0%)	16
Mice without tumour	7 (100%)	8 (57.1%)	1 (20.0%)	2 (25.0%)	10 (100%)	28
Total number of mice	7	14	5	8	10	44

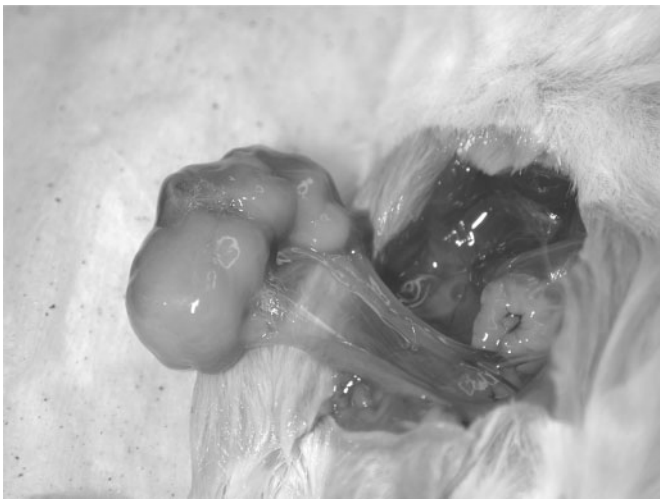

Figure 6. *In vivo* tumour formation in a W/W^{v} mouse, transplanted with a magnetic-activated cell sorting (MACS) (CD49f^{+}) and fluorescence-activated cell sorting (FACS) (CD49f^{+}, H-2K^{b-}) purified fraction.

transplantation could be conducted to screen the testicular tissue for malignant contamination before banking or transplanting the testicular stem cells back into the patient. However, apart from ethical considerations, this technique may prove controversial because of the theoretical risk of zoonosis in the graft.

In vitro expansion of spermatogonial stem cells might be another option (Kanatsu-Shinohara *et al*., 2003, 2005; Kubota *et al*., 2004). The use of a culture medium, specific for spermatogonial cells that do not support the growth and/or survival of the malignant cells, could be a promising technique for decontaminating testicular cell suspensions. This method could prove even more attractive because it could also solve the other major problem that needs to be overcome to obtain an effective transplantation, that is, the small number of germ cells within the testes and the small amount of tissue that can be obtained for preservation. When the transplantation of *in vitro*-cultured cells is intended, it will however be important to ensure that the culture medium does not contain any animal-derived proteins.

Other potential strategies for the improvement of the decontamination could comprise a spermatogonia-specific freezing protocol (Izadyar *et al*., 2002), the use of cancer cell-specific antibodies and complement treatment (Gee and Boyle, 1988) or the use of multiple cycles of cell sorting. Gribben *et al*. (1992) showed that all PCR-detectable lymphoma cells (1/10^{6} detection limit) could be purged out of bone marrow of non-Hodgkin's lymphoma patients after three cycles of treatment using a three or a four monoclonal antibodies cocktail followed by immunomagnetic bead depletion.

Although in our murine experiments, MACS and FACS showed enrichment of CD49f-positive cells; this enrichment did not result in a high number of transplanted testes with active spermatogenesis. The effectiveness of the transplantation was even lower than in our previous experiments in the same mouse model in which testicular cell suspensions of cryptorchid mice were used without further enrichment, that is, 64% of spermatogenesis in transplanted testes (Goossens *et al*., 2003). On the one hand, this apparent contradiction might be explained by a decreased viability and/or proliferation capacity of the cells after consecutive MACS and FACS treatment. Other groups (Shinohara *et al*., 1999, 2000) showed very encouraging transplantation results after MACS or FACS, but transplantation after combination of these two techniques has not before been reported. However, from our viability experiments, it seemed that the viability of the cells only slightly decreased (from 70.5% after digestion of the mouse testes to 60.5% after FACS) during the whole experiment. On the other hand, the cell suspensions after FACS contained a lot of cell debris, possibly making the suspensions more immunogenic than unsorted cells. The same may be the case with the frozen–thawed cell suspensions that did not give rise to recolonization after transplantation. In both cases, an immunological reaction may have killed donor cells (Rock *et al*., 2005).

How to overcome these problems? Finding a good freezing protocol for prepubertal testicular tissue or testicular cell suspensions and more specific for the testicular stem cells would mean a great benefit for fertility preservation and would certainly have a positive effect on further manipulation of the testicular stem cells (Izadyar *et al*., 2002; Kvist *et al*., 2006). To avoid an immunological reaction against the transplanted cells in the mouse experiments, another mouse model could be used. In our experiments, cells from donor mice with a different genetic background were transplanted to the recipients. The use of donor mice, genetically identical to the recipients except for the expression of a marker gene, might be a useful option. An extra step of Percoll gradient separation of the sorted cells could probably solve the problem of the cell debris in the sorted fraction.

The percentage of the patients at risk of losing their fertility as a result of sterilizing cancer therapies in which the testicular tissue may be contaminated with malignant cells is very uncertain. Yet, it would be very useful to try to determine the presence of malignant cells in the testicular tissue before cryopreservation. In this way, patients whose tissue is not contaminated could be transplanted without prior sorting of the cell suspensions. The PCR as described in this report might therefore be of great interest. The dilution sensitivity of 1/10 000 as obtained with this protocol is also used for the detection of minimal residual disease in lymphoblastic leukaemia (Dworzak and Panzer-Grümayer, 2003; Böttcher *et al*., 2004). The PCR protocol can even be improved by using patient-specific primers, encoding for the rearranged B- or T cell receptor of the malignant clone (Linke *et al*., 1997; Kerlan-Candon *et al*., 1998).

The number of patients in need of fertility preservation and whose testicular tissue is possibly contaminated by malignant cells is limited. Even so, decontamination will be inevitable for those patients at risk. FACS seemed very promising in preliminary murine studies. However, this technique will not be sufficient for a complete depletion of malignant cells, which may be a matter of life and death if used in a clinical application. Moreover, most cell sorting techniques entail an important cell loss; bearing in mind the limited number of stem cells available for transplantation, this creates feasibility problems. Therefore, screening of the testicular tissue for the presence of tumour cells before cryopreservation and/or transplantation is a major focus for research when the clinical application of the testicular stem cell transplantation is considered.

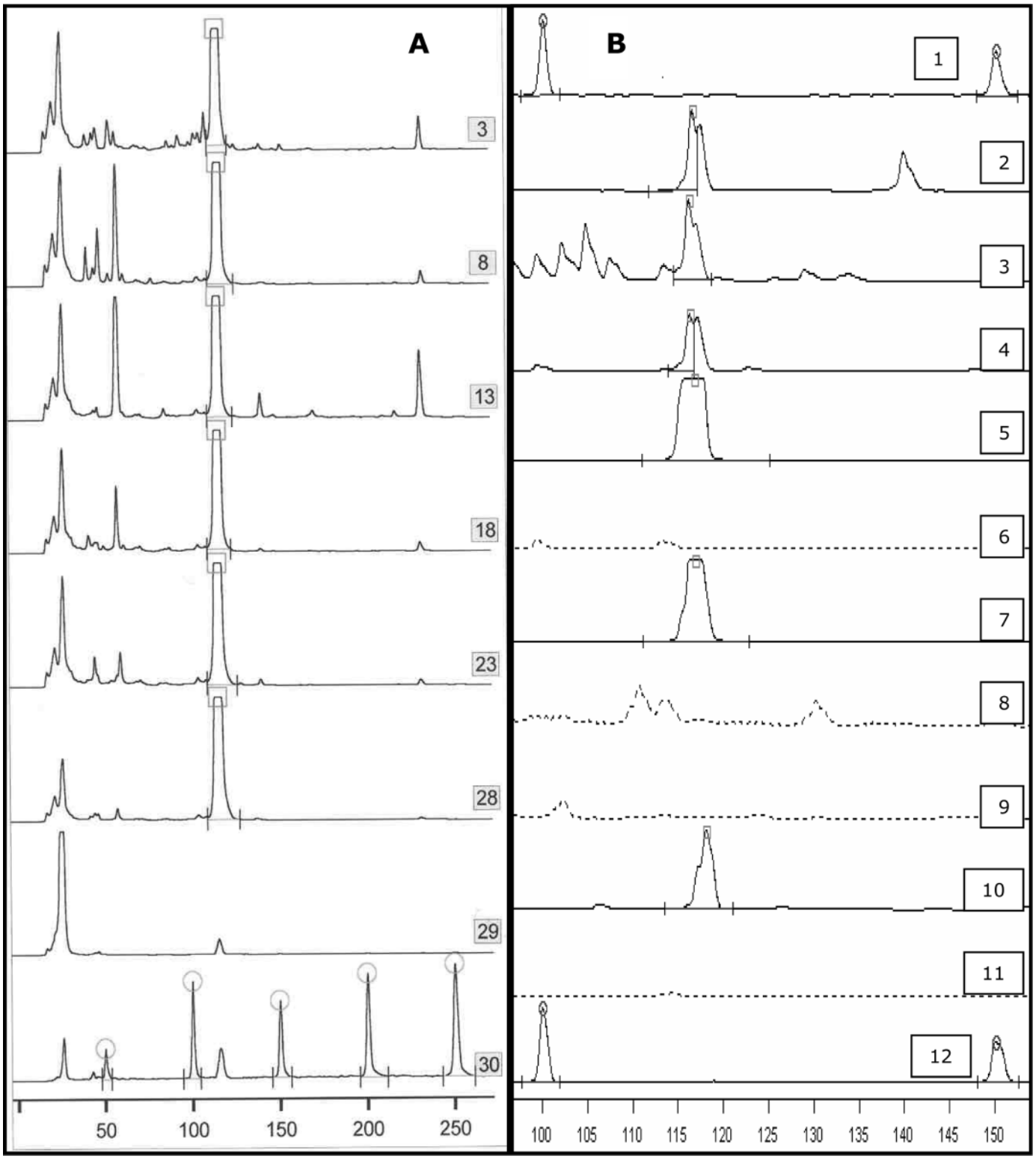

Figure 7. (**A**) PCR results for the patient samples contaminated with 5% SB cells. Lanes 3, 8, 13 and 18 are the samples of patients 2a, 3a, 4a and 5a, respectively. Lanes 23 and 28 are from DNA samples from pure SB cells. Lane 29 is the negative control (H_2O), and lane 30 contains an external standard with molecular weight (MW) markers 50–250 bp. (**B**) PCR results for the patient samples contaminated with 0.05% SB cells. A positive peak can be observed in lanes 2, 3, 4, 5 and 7 (respectively, patients 1b, 2b, 3b, 4b and 6b). Lane 6 contained the sample from patient 5b. Lanes 8, 9 (H_2O) and 11 (EL-4 cells) contain negative controls, and lane 10 comes from a DNA sample from a pure SB cell suspension. Lanes 1 and 12 contain an external standard with MW markers 50–250 bp.

Table III. Results of the human testicular suspensions, contaminated with 5% SB cells

Patient	HLA class I positivity (%) (Corrected for isotype control)		Tumour growth in culture (%)		PCR
	Before sort	After sort	Before sort	After sort	B cell
1a	12.03	0.09	100	0	[a]
2a	7.72	0.25	100	25	Positive
3a	16.30	2.25	100	50	Positive
4a	5.79	0.49	100	0	Positive
5a	17.14	0.88	100	50	Positive
Average (SD)	11.80 (± 5.04)	0.79 (± 0.87)	100 (± 0)	25 (± 25)	

[a]For patient 1a, no PCR could be performed. The testicular cell suspension of this patient was magnetic-activated cell sorted (MACS) ($CD49f^+$) and fluorescence-activated cell sorted (FACS) ($CD49f^+$, HLA class I^-). After this sort, there was not enough material left for a DNA extraction.

Table IV. Results of the human testicular suspensions, contaminated with 0.05% SB cells

Patient	HLA class I positivity (%) (Corrected for isotype control)		Tumour growth in culture (%)		PCR
	Before sort	After sort	Before sort	After sort	B cell
1b	6.42	0.32	100	50	Positive
2b	9.85	1.54	100	25	Positive
3b	5.64	0.02	75	50	Positive
4b	4.56	0.11	75	25	Positive
5b	8.41	0.00	100	0	Negative
6b	5.31	0.40	100	50	Positive
Average (SD)	6.70 (± 2.03)	0.40 (± 0.58)	91.7 (± 12.9)	33.3 (± 20.4)	

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