Decontamination of leukemic cells and enrichment of germ cells from testicular samples from rats with Roser’s T-cell leukemia by flow cytometric sorting

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Abstract

Testicular germ cell transplantation is a novel strategy for preservation of fertility in prepubertal cancer patients, but the risk of reseeding tumor cells into cured patients presently limits clinical application of this approach. To date, no systematic evaluation of the limitations of surface marker-based decontamination of testicular samples with acute lymphoblastic leukemia has been performed. Here, surface markers for leukemic (CD4 and major histocompatibility complex class I) and germ cells (epithelia cell adhesion molecule) in testicular samples infiltrated with Roser’s T-cell leukemia were identified. These markers were then used to delete leukemic cells and/or select for germ cells by flow cytometry (FACS). The resulting cell populations were analyzed by FACS, immunocytochemistry, or evaluation of leukemia transmission in syngeneic piebald variegated rats. Simple positive selection of germ cells or deletion of leukemic cells using specific surface markers was unable to effectively decontaminate testicular samples. The poor specificity of spermatogonial surface markers and aggregation of germ and leukemic cells limited the positive selection of germ cells, while immunophenotypic variation among lymphoblastic leukemia cells prevented adequate deletion of leukemic cells. Enzymatic treatment to disperse the testicular cells and feature of the intratesticular environment contributed to this immunophenotypic variation. Only germ cell selection in combination with leukemic cell deletion prevented leukemia transmission in association with intratesticular injection of the sorted cells. However, with such combined sorting, only 0.23% of the original testicular cells were recovered. With presently available techniques, flow cytometric purification of germ cells from a leukemic donor is not sufficiently effective or safe for clinical use.

Introduction

Isolation of spermatogonial stem cells (SSC) prior to and subsequent transplantation of these same cells back into the seminiferous tubules following intensive cancer therapy might be an effective approach to preventing damage to spermatogonial DNA and restoring the fertility of male survivors of childhood cancer. However, clinical application of this technique is presently limited by the risk that contamination of testicular samples might cause relapse in the otherwise cured patient (Jahnukainen et al. 2001).

Hematological spread of pediatric tumors results in a significant risk for intravascular and interstitial infiltration of testicular tissue by cancer cells. This is substantial in the case of acute lymphoblastic leukemia (ALL), where 20% of newly diagnosed patients exhibit microscopic infiltration of leukemic cells into their testes (Kim et al. 1981). Similar phenomena have been observed in association with experimental leukemia and lymphoma in rodents (Shaw et al. 1996, Jahnukainen et al. 2001).

An additional clinical concern is the relatively low number of stem cells present in the immature testis (Jahnukainen et al. 2006). Thus, in connection with the future development of clinical transplantation of germ cells, procedures designed to obtain testicular stem cell spermatogonia free from contaminating cancer cells and in good yield are urgently needed.

Flow cytometry (FACS) in association with selection on the basis of multiple parameters results in successful enrichment of murine SSC (Shinohara et al. 2000, Kubota et al. 2003, Ryu et al. 2004). The stem cells that are isolated in this fashion displayed low side-scatter (SSClow) and high forward-scatter (FCShigh) and expressed
epithelial cell adhesion molecule (Ep-CAM), α6-integrin/β1-integrin (CD49f), CD24, and thy-1 (CD90), but not v-integrin, c-kit, and major histocompatibility complex class I (MHC Cl I) on their surfaces (Shinohara et al. 1999, 2000, Giuili et al. 2002, Kubota et al. 2003, Ryu et al. 2004).

Promisingly, FACS has also recently been utilized to isolate germ cells from leukemic mice free from contamination by malignant cells (Fujita et al. 2005). In this case, cells of the myeloblastic leukemic (C1498) line, which cause leukemia in C57BL/6 mice, were eliminated from testicular cells removed from the leukemic donor on the basis of the expression of CD45 and MHC Cl I (Fujita et al. 2005). When testicular cells isolated in this manner were injected into the seminiferous tubules of healthy C57BL/6 mice, no transmission of leukemia was observed and the offspring originating from these cells were born healthy. More recently, the same surface markers were used successfully in seven of eight cases to remove cells of human leukemia and lymphoma lines from single-cell suspensions of human testicular cells with which the tumor cells had been mixed (Fujita et al. 2006).

In contrast, following positive selection for germ cells in human testicular samples employing the spermatogonial marker CD49f in combination with the removal of human B-cell acute lymphoblastic leukemic cells on the basis of their surface expression of HLA by FACS, the contamination by malignant cells was 0.58% in 10 of 11 samples sorted in this manner (Geens et al. 2007). This observation indicates that sorting by FACS is unlikely to be capable of completely depleting malignant cells from testicular samples.

To date, no systematic evaluation of the limitations associated with the removal of cancer cells from testicular samples on the basis of surface markers has been reported. In this context, it would be especially important to examine purification of testicular cell samples that have been infiltrated with leukemia cells by the natural spreading of this disease. Therefore, in the present investigation, Roser’s T-cell leukemia was employed to obtain infiltration of the testis by lymphoblasts and leukemic cells.

Roser’s leukemia is an acute T-lymphoblastic leukemia induced in piebald variegated (PVG) rats by irradiation and has been maintained by serial transmissions ever since its initial development (Dibley et al. 1975). This leukemia infiltrates immature testicular tissue (Jahnukainen et al. 1993) and the leukemic cells express the surface markers characteristic of immature T-lymphoblasts (Nestvold et al. 2004), properties that closely resemble those of human ALL. Following injection of more than 20 leukemic T-cells into a syngeneic rat, uninterrupted progression of leukemia occurs during a period of 14–19 days. The time of death is dependent on the number of cells injected, which allows this model to be used as a sensitive indicator of contamination of testicular samples by leukemic cells (Jahnukainen et al. 2001). The goals of the present study were to identify surface markers expressed specifically by leukemic or germ cells in testicular samples from rats with Roser’s leukemia and, thereafter, to employ these markers to delete leukemic cells from and/or select for germ cells in these samples by FACS. Special emphasis was placed on the identification of cellular factors that may limit the efficacy of such surface marker-based purification. Moreover, the yield of testicular cells and the time required for sorting were examined in order to evaluate the clinical feasibility of this approach.

Results

Immunophenotypic analyses of Roser’s rat T-leukemic cells and testicular cells

A summary of the immunophenotypic analyses of Roser’s rat T-leukemic cells (isolated from peripheral blood) and testicular cells from both leukemic and non-leukemic animals is presented in Table 1 and Fig. 1. Among the surface markers examined, Ep-CAM was the only one present on spermatogonia, but not on the leukemic cells. Thus, germ cells located at the basal membrane of seminiferous tubules exhibited strong positive staining for Ep-CAM (Fig. 1A), whereas antibodies toward this protein did not bind to leukemic cells in cytospin slides (Fig. 1B) or to such cells that had infiltrated into the interstitial compartment (Fig. 1A). MAB EE2 recognizing a glycoprotein expressed on the surface of mouse spermatogonia (Falcieri et al. 2004) was not found to react with the rat epitopes in the present study.

Altogether 97 and 99% of the lymphoblasts isolated from the peripheral blood of leukemic donor rats expressed CD4 and MHC Cl I respectively (Fig. 2A and C). The corresponding values were 15.8 and 39% for normal testicular cells (Fig. 2G and I) and 15.1 and 34.1% for leukemic testicular cells (Fig. 2D and F) respectively. An increase of 6.2 and 3.7% in the numbers of cells staining strongly for CD4 and MHC Cl I was exhibited by leukemic testicular cells in comparison with control testicular cells. With respect to CD45, 93% of the lymphoblasts from the peripheral blood of leukemic donors expressed this marker (Fig. 2B, M2, green curve), although at a lower level than CD4 and MHC Cl I (Fig. 2A and C), whereas leukemic and normal testicular cells exhibited no staining whatever for this surface marker (Fig. 2E and H; Table 1). Moreover, no expression of CD5 and CD43 by testicular cells could be detected, but leukemic lymphoblasts from the peripheral blood stained significantly for these markers (Table 1). In addition, leukemic cells in the interstitial tissue immuno-stained positively for CD4 and MHC Cl I, whereas there...
was no staining for these surface markers in the intratubular compartment (Fig. 1C and D).

On the basis of these findings, the expression of both CD4 and MHC Cl I was employed to identify Roser’s rat T-lymphoblasts in testicular samples, and expression of Ep-CAM was utilized as a marker for non-leukemic testicular cells. In FACS analyses, CD4- and MHC Cl I-positive leukemic cells displayed SSC low and FSC high (data not shown), a pattern which overlapped with that of the Ep-CAM-positive cells in leukemic testicular samples.

Table 1 Immune-phenotypical analysis of Roser’s leukemic T-cells and normal and leukemic testicular cells from rats employing immunocytochemical staining or flow cytometry (FACS).

<table>
<thead>
<tr>
<th>Surface markers</th>
<th>Leukemic cells</th>
<th>Normal testicular cells</th>
<th>Leukemic testicular cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immunocytochemical staining (% of cells labeled)a</td>
<td>FACS</td>
<td>Immunocytochemical staining (% of cells labeled)a</td>
</tr>
<tr>
<td>Lymphoid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>2 ± 0.5</td>
<td>0.8 ± 0.2</td>
<td>–</td>
</tr>
<tr>
<td>CD4</td>
<td>99 ± 0.1</td>
<td>97 ± 0.2</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>CD90</td>
<td>98 ± 0.3</td>
<td>93 ± 0.4</td>
<td>2.8 ± 0.0</td>
</tr>
<tr>
<td>MHC Cl I</td>
<td>99 ± 0.4</td>
<td>100 ± 0.0</td>
<td>11.6b</td>
</tr>
<tr>
<td>CD8</td>
<td>–</td>
<td>79 ± 1.6</td>
<td>89b</td>
</tr>
<tr>
<td>CD5</td>
<td>–</td>
<td>68b</td>
<td>–</td>
</tr>
<tr>
<td>CD43</td>
<td>–</td>
<td>96b</td>
<td>–</td>
</tr>
<tr>
<td>CD45</td>
<td>–</td>
<td>93b</td>
<td>–</td>
</tr>
<tr>
<td>Spermatogonial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RET</td>
<td>97 ± 0.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α6/β1 Integrin</td>
<td>100 ± 0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ep-CAM</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>6.8 ± 0.2</td>
</tr>
</tbody>
</table>

–, not analyzed.

aMean ± S.E.M. (n = 4). bOnly one sample studied.

was no staining for these surface markers in the intratubular compartment (Fig. 1C and D).

On the basis of these findings, the expression of both CD4 and MHC Cl I was employed to identify Roser’s rat T-lymphoblasts in testicular samples, and expression of Ep-CAM was utilized as a marker for non-leukemic testicular cells. In FACS analyses, CD4- and MHC Cl I-positive leukemic cells displayed SSC low and FSC high (data not shown), a pattern which overlapped with that of the Ep-CAM-positive cells in leukemic testicular samples.

**Figure 1** Representative light micrographs illustrating that (A) spermatogonia located at the basement membrane of seminiferous tubules stained positively for Ep-CAM (brown circles), whereas leukemic cells in the interstitium (arrow in A) and on cytospin slides (B) remained unstained. Only the leukemic cells that had infiltrated into the testicular interstitium (arrow heads) and blood vessels (arrows) stained positively for CD4 (C) and MHC Cl I (D) antibodies. (E) In a cytospin sample, CD4-positive leukemic cells were seen to be attached to germ cells (arrow) selected for on the basis of their expression of Ep-CAM. (F) Aggregation of testicular and leukemic cells was also detected when leukemic cells (left insert, LC) emitting green fluorescence (PKH26) were mixed with testicular cells (right insert, GC) emitting red fluorescence (PKH67). Furthermore, when cells displaying the germ cell phenotype, together with low side-scatter (SCC low) and high forward-scatter (FSC high), were gated in area R1 (G) and analyzed by FACS (H), 2.4% of these cells stained positively for both leukemic (CD4 and MHC Cl I) and germ cell (Ep-CAM) surface markers, indicating the presence of cell aggregates. Bar = 20 μm.

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and was comparable with the scattering characteristics of spermatogonia reported earlier (Shinohara et al. 1999, 2000, Kubota et al. 2003).

**Cell sorted by FACS: transmission of leukemia, phenotypic analysis, and yield**

**Protocol 1 (Ep-CAM<sup>-Alexa</sup>)**

One of the rats that received testicular cells from a leukemic donor that were selected for on the basis of their expression of Ep-CAM<sup>-Alexa</sup> did not develop leukemia, whereas the other two died 24 and 33 days after transplantation of these cells (Fig. 3). As summarized in Table 2, no cells with the leukemic CD4<sup>+</sup>/MHC Cl I<sup>+</sup> phenotype were detected in these samples. Altogether, 5.1% of the cells expressed both leukemic CD4<sup>+</sup> and MHC Cl I<sup>+</sup> phenotype. In comparison with normal testicular cells, 5% of more leukemic testicular cells demonstrated strong expression of CD4<sup>+</sup> and MHC Cl I<sup>+</sup>, while 6.2 and 3.7% of more leukemic testicular cells demonstrated weak expression of CD4<sup>+</sup> and MHC Cl I<sup>+</sup>, respectively. Indeed, this conclusion is supported by our observation in immunostained cytospin slides of a CD4<sup>+</sup>-positive leukemic cell that had aggregated with an Ep-CAM-positive testicular cell (Fig. 1E).

1.1% of the cells selected on the basis of their surface expression of Ep-CAM also expressed CD90, another spermatogonial marker, a value that was the same as the one before sorting (1.0%). Only 0.93 million of 110 million cells subjected to sorting were recovered, i.e., more than 99% of the cells were lost during the 2-h period associated with this sorting procedure (Table 3).

**Protocol 2 (Ep-CAM<sup>+</sup>2nd Ab<sup>-PE</sup>)**

FACS selection of testicular cells following labeling with primary antibodies toward Ep-CAM and secondary fluorescent antibodies was not more effective with regard to purification or survival of the recipient animals than protocol 1 (Fig. 3). Moreover, no difference in the survival of recipients injected with two distinct subpopulations of germ cells as described earlier by Ryu et al. (2004), expressing low and high levels of Ep-CAM was observed.
Repitition of this same experiment employing a higher concentration of germ cells and more rapid sorting resulted in a prolongation of the sorting time and a reduction in the viability of the testicular cells obtained (Table 3, experiment 2). Moreover, no improvement in purification was achieved when Ep-CAM-high-positive cells were sorted a second time using the more narrow gating area R2 (Fig. 4, D2; Table 3, experiment 3). After FACS sorting, the proportion of the cells that expressed both of the spermatogonial surface markers CD90 and Ep-CAM was 0.8% (Table 2).

Protocol 3 (CD4−PE + MHC Cl I−PE)

All of the rats injected with preparations from which cells expressing both of the leukemic cell markers CD4 and MHC-Cl I had been deleted died only a few days later than animals that received unsorted leukemic testicular cells (Fig. 3). Neither the length of survival nor the course of leukemia development was altered when the gate for deletion by FACS was enlarged by reducing the threshold for identification of CD4- and MHC Cl I-positive cells (Fig. 4B, gate R3, and Fig. 3). Cell preparation obtained in this way revealed 0.2% of the cells expressing the leukemic markers CD4 and MHC Cl I, and the same proportion expressed both of the spermatogonial markers CD90 and Ep-CAM (Table 2). A relatively high number of cells (2.1 and 1.3 × 10^6 respectively; Table 3, experiments 1 and 2) were retrieved after this 2-h sorting procedure.

Protocol 4 (Ep-CAM+Alexa + CD4−PE/MHC-1−PE)

When testicular cells prepared both by positive selection and by negative deletion were injected into PVG rats, both recipients survived for at least 4 months without developing leukemia (Fig. 3). As documented in Table 2, no cells expressing the leukemic markers CD4, MHC Cl I, or CD90 could be detected after sorting in this manner. In contrast, when the cells were used after only one sorting cycle, all five recipients died within 20.6 ± 0.40 days after injection (data not shown). In total, 0.6% of the cells recovered with this procedure expressed both of the spermatogonial markers CD90 and Ep-CAM. Unfortunately, only 0.7 × 10^6 of the original 300 × 10^6 testicular cells subjected to this 4-h procedure were recovered (Table 3, experiment 2).

Immunocytochemical identification of testicular cells

Table 4 summarizes the results of the immunocytochemical analyses performed on the cell preparations sorted by FACS according to the various protocols. All the preparations selected on the basis of Ep-CAM expression included a high percentage of Oct-4-positive cells, with direct labeling with antibodies toward this surface marker.
yielding the purest fraction of such cells. Deletion of cells expressing CD4 and MHC Cl I produced a heterogeneous population of testicular cells, 5% of which expressed GATA-4. Examination under the light microscope revealed that many of these GATA-4-positive cells were elongating spermatids (data not shown).

Aggregation of leukemic cells and testicular cells

Leukemic cells labeled with a fluorescent dye were seen to aggregate with testicular cells labeled with a different fluorescent dye (Fig. 1 F). Upon incubation of 100 testicular cells per leukemic cell for 1, 2, and 3 h, the mean numbers of aggregates obtained per 100 cells examined were 1.1 (±0.31), 1.8 (±0.33), and 1.3 (±0.28) respectively. With the lower ratio of 10:1, the corresponding values were 2.7 (±0.83), 2.5 (±0.28), and 2.7 (±0.19). Thus, neither the length of the period of incubation nor the relative number of leukemic cells appeared to exert any pronounced influence on the formation of aggregates. When leukemic cells were labeled with antibodies toward Ep-CAM, CD4, and MHC Cl I, and the gate for sorting of cells exhibiting the SSClow and FSChigh characteristic for spermatogonia by FACS (Fig. 1G, R1), 2.4% of the total cells recovered in this area were found to be positive for all three of these markers (Fig. 1H, upper right quadrant). We assume that the majority of these cells were aggregates of germ and leukemic cells.

Discussion

Transplantation of autologous testicular germ cells is one approach to the preservation of future fertility in prepubertal cancer patients whose treatment might otherwise result in sterility. The major limitation in this context is the risk of reseeding tumor cells into patients who have been cured. On the basis of promising findings with the mouse and cell lines, sorting by FACS has been proposed as a potential removal of tumor cells from testicular samples (Rijnsburger et al., 2005, 2006). However, the efficacy of such selection is entirely dependent on the availability of specific surface markers for cancer and/or normal testicular cells. In the present investigation, poor specificity of the markers for leukemic and germ cell lines sorting by FACS has been proposed as a potential removal of tumor cells from testicular samples (Rijnsburger et al., 2005, 2006).

Table 2: A representative flow cytometry (FACS) analysis of normal and leukemic testicular cells before and after sorting by FACS.

<table>
<thead>
<tr>
<th>Phenotype of cell fraction</th>
<th>Identity of cell fraction</th>
<th>Testicular cells from control rats (% of total)</th>
<th>Pre-sorting</th>
<th>Post-sorting according to Protocol 1 (Ep-CAM&lt;sup&gt;−&lt;/sup&gt; + PE&lt;sup&gt;+&lt;/sup&gt; + MHC Cl I&lt;sup&gt;+&lt;/sup&gt; + Ep-CAM&lt;sup&gt;−&lt;/sup&gt;)</th>
<th>Post-sorting according to Protocol 2 (Ep-CAM&lt;sup&gt;−&lt;/sup&gt; + PE&lt;sup&gt;+&lt;/sup&gt; + MHC Cl I&lt;sup&gt;+&lt;/sup&gt; + Ep-CAM&lt;sup&gt;−&lt;/sup&gt;)</th>
<th>Post-sorting according to Protocol 3 (CD4&lt;sup&gt;−&lt;/sup&gt; + PE&lt;sup&gt;+&lt;/sup&gt; + MHC Cl I&lt;sup&gt;+&lt;/sup&gt; + Ep-CAM&lt;sup&gt;−&lt;/sup&gt;)</th>
<th>Post-sorting according to Protocol 4 (CD4&lt;sup&gt;−&lt;/sup&gt; + PE&lt;sup&gt;+&lt;/sup&gt; + MHC Cl I&lt;sup&gt;+&lt;/sup&gt; + Ep-CAM&lt;sup&gt;−&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4- and MHC Cl I- positive Ep-CAM-negative</td>
<td>Leukemic cells + normal testicular lymphocytes</td>
<td>11.3</td>
<td>16</td>
<td>0 (±0.2)</td>
<td>0 (±0.1)</td>
<td>0 (±0.1)</td>
<td>0 (±0.1)</td>
</tr>
<tr>
<td>CD4- and MHC Cl I- positive Ep-CAM-positive</td>
<td>Aggregates of germ and leukemic cells + testicular somatic cells</td>
<td>1.5</td>
<td>0.5</td>
<td>5.1 (±0.3)</td>
<td>0.2 (±0.1)</td>
<td>0.2 (±0.1)</td>
<td>0.2 (±0.1)</td>
</tr>
<tr>
<td>CD90- and Ep-CAM-positive CD4- and MHC Cl I- negative</td>
<td>Spermatogonia stem cells</td>
<td>0.5</td>
<td>1.0</td>
<td>1.1 (±0.1)</td>
<td>0.8 (±0.1)</td>
<td>0.2 (±0.1)</td>
<td>0.6 (±0.1)</td>
</tr>
<tr>
<td>CD4-, MHC Cl I- and CD90- positive Ep-CAM-negative</td>
<td>Putative leukemic cells + testicular somatic cells</td>
<td>7.7</td>
<td>12.3</td>
<td>0.5 (±0.1)</td>
<td>0.1 (±0.1)</td>
<td>0 (±0.1)</td>
<td>0 (±0.1)</td>
</tr>
</tbody>
</table>

<sup>−</sup>, not analyzed.
evaluated, aggregation of germ and leukemic cells, and heterogeneity of the leukemic cell population were found to seriously impair the efficiency of purification of testicular samples by FACS. Positive selection of germ cells or deletion of leukemic cells alone was insufficient to decontaminate testicular cell preparations, in agreement with previous observations (Geens et al. 2007). Only the combination of positive selection and deletion prevented transmission of leukemia in association with testicular cell transplantation in rats.

Here, detailed analysis of four known surface markers for spermatogonia revealed that poor specificity seriously limits their efficacy in separating germ cells from leukemic lymphoblasts. To date, only a few surface markers that can be used to identify the spermatogonial subpopulation with stem cell potency have been identified (Shinohara et al. 2000, Viglietto et al. 2000, Falciatori et al. 2004, Kanatsu-Shinohara et al. 2004). Of these markers, RET, a $6/b_1$ integrin, and CD90 were detected here on the surface of rat leukemic T-lymphoblasts as well.

These observations strongly suggest that leukemic lymphoblasts and spermatogonia share stem cell-like characteristics, making their separation highly difficult. Indeed, Ep-CAM was the only spermatogonial marker not detected on the surface of Roser’s rat leukemic lymphoblasts. Ep-CAM, a calcium-independent homophilic adhesion molecule expressed by most epithelia and carcinoma (Litvinov et al. 1997), has recently been utilized to isolate murine gonocytes and type A spermatogonia for culture (van der Wee et al. 2001, Moore et al. 2002).

Although considered to be a marker for SSC, our present immunohistochemical examination revealed staining for Ep-CAM in a continuous ring of cells on the basement membrane, without changes in intensity or differences between seminiferous tubules (Fig. 1A). This observation, which is consistent with an earlier report (Anderson et al. 1999; Fig. 2C), suggests that Ep-CAM is not a unique marker for SSC in the mature rat testis, but is probably expressed by germ cells in general. The patterns of expression of Ep-CAM in the immature and adult rat testis may differ significantly, since 90% of the germ cells from rat pup testis exhibiting SSClow and FSC-high and expressing Ep-CAM demonstrated stem cell potential in a functional assay involving germ cell transplantation. This latter fraction was also found to express Thy-1 (CD90), a marker for a variety of stem cells, including hematopoietic and SSC (Baum et al. 1992, Ryu et al. 2004).

In the present study, sorting based on a combination of FSC-high and SSC-low together with expression of Ep-CAM (protocols 1, 2, and 4, Fig. 4) yielded cells which expressed Ep-CAM (data not shown) and also Oct-4 (Table 4; Hofmann et al. 2005). The cell population obtained with protocol 1 contained no cells that expressed the leukemic makers CD4 and MHC Cl I. However, a distinctive cell

<table>
<thead>
<tr>
<th>Table 3 Summary of the experimental procedures employed.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of cells recovered</strong> (×10⁶)</td>
</tr>
<tr>
<td><strong>Cell viability (%)</strong></td>
</tr>
<tr>
<td><strong>Sorting time (h)</strong></td>
</tr>
<tr>
<td><strong>Number of testicular cells used for sorting</strong> (×10⁶)</td>
</tr>
<tr>
<td><strong>Procedure for labeling of the cells</strong></td>
</tr>
<tr>
<td><strong>Experiment number</strong></td>
</tr>
<tr>
<td><strong>Protocol 1</strong> (Ep-CAMAlexa)</td>
</tr>
<tr>
<td><strong>Protocol 2</strong> (Ep-CAMC2nd AbPE)</td>
</tr>
<tr>
<td><strong>Protocol 3</strong> (CD4PE MHC IPE)</td>
</tr>
<tr>
<td><strong>Protocol 4</strong> (Ep-CAMAlexaCD4PE/MHC-1PE)</td>
</tr>
<tr>
<td><strong>Number of donors/testes used</strong></td>
</tr>
<tr>
<td><strong>Number of recipients injected</strong></td>
</tr>
<tr>
<td><strong>Cell fraction injected</strong></td>
</tr>
</tbody>
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population expressing markers for both leukemic and germ cells was detected, and two of the three recipients injected with the cells from this protocol died from relapsing leukemia (Table 2).

This finding indicated the presence of leukemic cells that had aggregated with Ep-CAM-positive cells, an explanation that was confirmed by our observation of CD4-positive cell aggregates in immunostained cytopsin slides. Rapid aggregation that was independent of the ratio of normal testicular to leukemic cells also occurred when these two types of cells (labeled with different fluorescence dyes) were mixed together (Fig. 1F). In this case, the cell aggregates formed displayed SSClow and FSChigh and were located in the gated FACS containing germ cells. It appears highly likely that adhesion of this sort represents a serious risk for contamination of all germ cells separated on the basis of surface markers by malignant cells. Deletion of leukemic cells on the basis of their surface markers is mandatory to eliminate such contamination.

On the other hand, removal of leukemic cells on the basis of their specific surface markers did reduce the contamination of testicular cell preparations, but to a lesser extent than that achieved by positive selection on the basis of Ep-CAM. None of the recipients of the former cell preparations survived. This result is very different from that described by Fujita et al. (2005) who used negative selection by FACS based on one leukemia specific together with one somatic cell marker (MHC Cl I) to obtain total decontamination of leukemic testicular samples.

One obvious reason for these different outcomes is the difference in the models employed for leukemia. The myeloblastic leukemic (C1498) cells studied by Fujita et al. (2005) uniformly express CD45 and MHC Cl I.

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Table 4 Immunocytochemical analysis and detection of alkaline phosphatase and 3β-hydroxysteroid dehydrogenase (HSD) in the cell preparations isolated by flow cytometry (FACS) according to protocols 1–4:

<table>
<thead>
<tr>
<th>Sorting procedure</th>
<th>Oct-4</th>
<th>GATA-4</th>
<th>ED1</th>
<th>Alkaline phosphatase</th>
<th>3β-HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 1 (Ep-CAMAlexa)</td>
<td>96.5±1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Protocol 2 (Ep-CAM+2nd AbPE)</td>
<td>96.0±1.5</td>
<td>0.6</td>
<td>0.2</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>Protocol 3 (CD4PE+MHC Cl IPE)</td>
<td>1.8±0.2</td>
<td>5.0</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Protocol 4 (Ep-CAMAlexa+CD4PE+MHC Cl IPE)</td>
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The values presented are mean±S.E.M. for three independent determinations in the case of Oct-4 and single determinations in all other cases.
l on their surface, whereas variations in leukemic cell immunophenotype drastically limited the efficacy of negative selection in our study. Roser's rat leukemic T-cells were found to express a number of lymphoid markers, including CD3, CD4, CD8, CD45, and CD43, but there was a significant immunophenotypic variation, especially with respect to the markers expressed at low levels, CD8 and CD45 (Table 1). In the clinical situation as well, immunophenotypic variation and formation of subsets of leukemic cells occur regularly in connection with the development of human acute leukemias (Lay et al. 1971, Catovsky et al. 1974).

Moreover, we also demonstrate unequivocally here that the testicular micro-environment may contribute to immunophenotypic variation among testicular leukemic cells in this organ. The leukemic surface markers CD45, CD43 and CD5 could not be detected in testicular samples in which 4% of the cells were leukemic, despite the fact that these markers were expressed on lymphoblasts in the peripheral blood of the same animals. In addition, heterogeneous expression of surface markers on testicular leukemic cells was also detected in our previous study on magnetic cell sorting involving the same model system (Hou et al. 2007b). In that case, weakly CD4-positive leukemic lymphoblasts were recovered in the flow-through fraction, whereas strongly CD4-positive leukemic cells were deleted by the magnetic cell sorting. Either the leukemic lymphoblasts alter their expression of surface markers after infiltrating testicular tissue or only a specific leukemic subclone infiltrates the testis.

Our observation that enzymatic dispersion decreases the fluorescence intensity of labeled leukemic cells suggests that use of such a procedure to prepare testicular cells may also result in digestion of surface marker proteins. Here, the fluorescence intensity of leukemic cells labeled with antibodies toward CD4, but not with antibodies toward the weak markers CD5, CD45 or CD43, was reduced by enzymatic dispersion, indicating that our inability to detect these latter markers in our testicular cell samples was not due to sample preparation.

The tissue- and preparation-dependent expression of leukemic cell surface markers observed here raises serious questions concerning the relevance of certain previous purification studies (Fujita et al. 2006, Geens et al. 2007). Thus, FACS sorting of artificial mixtures of cancer cell lines and normal testicular cells may not reflect the clinical situation, since the malignant cells employed do not originate from testicular tissue and have not been exposed to enzymatic digestion. In addition, the present findings suggest that surface markers expressed on leukemic cells in peripheral blood will probably not be suitable for clinical FACS sorting of leukemic testicular samples.

Only by employing positive selection of germ cells in combination with deletion of leukemic cells did we succeed in isolating testicular cells free from leukemic cells. No testicular somatic cells were detected in such preparations and all rats injected with them survived. The results obtained by Geens in a previous comparable investigation were less promising. In that case, after one cycle of combined positive selection and deletion, 0.4 and 0.6% contamination by murine lymphoma and human acute B-lymphoblastic leukemia cells respectively was detected (Geens et al. 2007). However, these findings by Geens are strikingly similar to ours after only one cycle of purification by FACS, where all recipients of such cells developed leukemia. A second sorting cycle was required to abolish the contamination by leukemic cells, suggesting that repeated FACS cycles may enhance the efficacy of testicular cell sorting.

One clear disadvantage associated with the use of a second sorting cycle was the serious loss of cells. Thus, only $0.7 \times 10^6$ of the initial $300 \times 10^6$ cells sorted were recovered. This low yield of 0.23%, together with the lack of a specific marker for monitoring donor-derived spermatogenesis, prevented us from confirming the stem cell capacity of the isolated cells by germ cell transplantation. Nor could we evaluate the risk of leukemia relapse associated with injection of larger numbers of testicular cells. When $0.1 \times 10^6$ testicular cells purified in this manner were injected here, no leukemia was detected, but injection of a 10- to 100-fold greater number of cells would have resulted in the transmission of leukemia. One major limiting factor in connection with assessment of cell purification by FACS may be the relatively low ability of this approach to detect contaminating cancer cells, which is at best one cell among $10^3$--$10^5$ cells sorted (Hu et al. 2005).

The cells expressing both CD90 and Ep-CAM, but no leukemic surface markers, detected here may be SSC (Ryu et al. 2004). The level of these cells in our testicular samples (0.5--1.1%) was well within the low level of SSC described previously in the rodent testis (Tegelenbosch & de Rooij 1993). The proportion of CD90- and Ep-CAM-positive cells was the highest in the unsorted testicular cell preparations, indicating that none of the sorting protocols applied here was capable of selection for these putative SSC. Only 3000–9000 CD90- and Ep-CAM-positive cells could be collected from 6 to 10 adult rat testes by the procedures employed here.

Morphological studies indicate that one testis of a 10-year-old prepubertal boy contains $\sim 83 \times 10^6$ germ cells, with the corresponding value for a boy <1 year of age being $13 \times 10^6$ germ cells (Muller & Skakkebaek 1983). It therefore seems unlikely that small testicular biopsies from young boys will provide a sufficient number of cells for combined positive selection and deletion by FACS.

Our present use of experimental lymphoblastic leukemia as a model provides the first insights concerning the limitations associated with surface marker-based selection of testicular cells for clinical transplantation in
case of ALL. Patients with this disease present the largest pediatric group that could benefit from novel strategies designed to assure future fertility by germ cell transplantation. Here, the poor specificity of spermatogonial surface markers, aggregation of germ and leukemic cells, and significant variations in the expression of specific leukemic surface markers were found to seriously limit the efficacy of both positive selection of normal testicular cells and deletion of leukemic testicular cells by FACS. Future development of functional deletion of malignancy, i.e., by culturing in vitro or xenotransplantation into an intermediate host (Hou et al. 2007a), may provide new tools to guarantee the absence of tumor cells from clinical samples of testicular cells. The present investigation helps provide a platform for planning such future studies.

Materials and Methods

Transmission of T-cell leukemia and preparation of the cells

Transmission of leukemia and isolation of leukemic lymphoblasts (from lymph nodes and blood) and testicular cells were performed as described previously (Jahnukainen et al. 2001, Hou et al. 2007a). A total of 40 terminally leukemic PVG rats 40 days of age (with transmission of leukemia occurring when they were 25 days old) and 3 age-matched, nonleukemic rats served as donors of lymphoblasts, testicular cells, and/or testicular sections (stored frozen; see below). As evaluated by trypan blue exclusion, the viability of cells prepared in this manner was routinely 85–97%, and these cells were maintained on ice prior to initiation of the experiments.

The lymphoblasts utilized for examination of surface markers, cell aggregation, and immunophenotypic variation were isolated from the enlarged cervical lymph nodes (Jahnukainen et al. 2001) and on Ficoll from peripheral blood of terminally ill leukemic rats. Cells obtained from the peripheral blood or testis were labeled shortly after isolation either with antibodies for FACS analysis (see below) or with fluorescence dyes for evaluation of cell adhesion or else used to make cytospin slides for immunocytochemical analysis (see below). For the preparation of frozen sections, the testes removed from three healthy and three leukemic rats at 40 days of age were immersed in a solution of OCT (HistoLab, Västra Frölunda, Sweden), frozen in dry ice, and stored at −70°C. The frozen testes were cut into 12 μm thick sections with a microtome, mounted on Superfrost slides, and maintained thereafter at −70°C until use.

Screening for surface markers that could potentially allow efficient sorting by FACS

Lymphoblasts obtained from leukemic donors or testicular cells from control animals were first incubated with mouse anti-rat CD5, CD43 and CD45 antibodies (kindly supplied by Dr Olle Lidman at Karolinska Institute, Stockholm, Sweden) at a dilution of 1:100 (using 10 μl/10^6 cells) for 30 min; then washed twice with PBS containing 0.5% fetal calf serum (FCS; designated hereafter as PBS/FCS); and thereafter incubated with PE-goat anti-mouse F(ab')2 (diluted 1:20, 10 μl/10^6 cells; DakoCytomation, Glostrup, Denmark) for 30 min. Following two additional washes with PBS/FCS, these cells were subjected to FACS analysis. For direct labeling, the cell preparations were incubated instead with mouse anti-rat CD3−PE (5 μl/10^6 cells), CD4−PE (5 μl/10^6 cells), CD90−PerCP (10 μl/10^6 cells), MHC Cl I−PE (5 μl/10^6 cells; all purchased from BD Biosciences, San Jose, CA, USA), CD8−FITC (10 μl/10^6 cells; Serotec, Oxford, UK), or Ep-CAM−Alexa (0.4 μl/10^6 cells; BioVendor Gmbh, Heidelberg, Germany) antibodies for 30 min; washed twice with PBS/FCS; and subsequently subjected to FACS analysis. As negative controls, identical cell preparations were incubated with irrelevant mouse IgG1-PE (Chemicon, Boronia, VIC, Australia), IgG1-FITC (Chemicon), IgG1-Alexa 488 (Serotec), and IgG1−PerCP (BD Biosciences) antibodies respectively at the same concentrations. Gating for FACS analysis was designed to exclude dead cells, cell debris, and granulocytes and a FACS Calibur flow cytometer (BD Bioscience) employing Cell quest Pro acquisition software (BD Bioscience) was used.

Cell sorting

For purposes of cell sorting, four FACS sorting protocols were designed (Fig. 4). Sorting was carried out following labeling of testicular cells with fluorescence antibodies (Fig. 4). For protocols 1, 3, and 4, testicular samples were incubated directly with Ep-CAM−Alexa (0.4 μg/10^6 cells; BioVendor) or CD4−PE and MHC-Cl I−PE (5 and 10 μl/10^6 cells respectively; BD Biosciences) or with all three antibodies for 30 min; thereafter washed twice with cold PBS/FCS containing 2 mM EDTA; and then diluted to a concentration of 15×10^6 to 20×10^6 cells/ml for subsequent passage through a filter with pores 40 μm in diameter. In order to amplify the labeling signal, an indirect labeling procedure was performed, which was designated as protocol 2 (Fig. 4). In this case, testicular cells were first incubated with primary mouse anti-rat Ep-CAM antibodies (0.1 μg/12×10^6 cells; BioVendor) for 30 min and, following two washes with PBS, further incubated with PE-conjugated goat anti-mouse secondary antibody (10 μl/10^6 cells; DakoCytomation) for 30 min, followed by two more washes with PBS.

Following the cell labeling, the testicular cells displaying spermatogonial characteristics (Ryu et al. 2004) with SSC[low] and FSC[low] were subjected to FACS sorting according to four different protocols as described in Fig. 4. In the case of protocol 1, cells labeling positively for Ep-CAM−Alexa in gate R1 (Fig. 4A) were collected. In protocol 2, testicular cells labeled with both antibodies toward Ep-CAM and secondary PE-antibodies (Ep-CAM+2nd Ab−PE) in gates Ep-CAM−high and Ep-CAM−low (Experiments 1 and 2, Table 3) and R2 (Experiment 3, Table 3) were collected and analyzed separately (Fig. 4, D1 and D2). In protocol 3, cells expressing CD4−PE and MHC Cl I−PE in gate R3 (Fig. 4B) were deleted, while the unlabeled testicular cells in area R4 were collected. Finally, cells treated according to protocol 4 were sorted by a...
combination of the approaches used in connection with protocols 1 and 3. Accordingly, cells expressing CD4 and MHC Cl I in gate R6 (Fig. 4C) were deleted, and cells expressing Ep-CAM in gate R5 (Fig. 4C) were simultaneously selected. All sorting was performed on a MoFlo high-speed cell sorter (DakoCytomation) using two cycles, except that only one sorting cycle was carried out for experiments 1 and 2 in association with protocol 2 (Table 3).

**Evaluation of the sorting procedures**

The testicular cell subpopulations obtained with protocols 1–4 were either used for transplantation studies (see below) or further labeled for additional FACS analysis. In the case of FACS analysis, 0.3 × 10^6 to 0.5 × 10^6 Ep-CAM^+ cells obtained by protocol 1 were labeled with CD4^{−PE}, MHC Cl I^{−PE}, and CD90^{−PerCP} antibodies, while the cells from protocols 2 and 4 were labeled with CD90^{−PerCP} antibodies. The CD4/MHC Cl I-negative cells collected following protocol 3 were labeled with CD90^{−PerCP} and Ep-CAM^{−Alexa} antibodies as described above. As apresorting control, testicular cells from leukemic rats and equal number of cells from control animals were placed immediately after isolation into three separate tubes (10^6 cells per tube) and subsequently labeled as follows: tube 1: Ep-CAM^{−Alexa}, CD4^{−PE}, and MHC Cl I^{−PE} antibodies; tube 2: Ep-CAM^{−Alexa} and CD90^{−PerCP} antibodies; and tube 3: CD4^{−PE}, MHC Cl I^{−PE}, and CD90^{−PerCP} antibodies (at concentrations 0.4 μg, 5 μl, 5 μl, and 10 μl per 10^6 cells respectively). The phenotype of cell fractions that are labeled with these antibodies was analyzed by FACS (Table 2). In all cases, at least 20 000 cells from each sample were analyzed by FACS. In addition, cytospin slides prepared from these samples were analyzed immunocytochemically (see below).

**Testicular cell injection**

For the transplantation studies, 25 syngeneic PVG rats at 40 days of age were injected intratesticularly (into the left testis) with 0.1 × 10^6 testicular cells sorted according to one of the four protocols (Table 3). As a positive control for transmission of leukemia, six animals received the same number of FACS-sorted CD4- and MHC Cl I-positive leukemic cells intratesticularly. Furthermore, three rats received the same number of unsorted cells as a standard control. Animals that survived for more than 120 days after transplantation were considered as being non-leukemic.

**Immunocytochemical staining**

The cryopreserved cytospin slides and testicular sections were air-dried at room temperature (RT) for 10 min; fixed in methanol/acetone (1:1) at −20°C for 10 min; washed twice with PBS; and then incubated overnight at 4°C with antibodies against the lymphoid markers CD3-ζ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD4 and z/βTCR (Biosource, Camarillo, CA, USA), CD90 (Abcam, Cambridge, UK), and MHC Cl I (supplied by Dr Olle Lidman), all at dilutions of 1:100; or antibodies against the spermatogonial surface markers Ep-CAM (Ryu et al. 2004; BioVendor), α6/β1 integrin (Shinohara et al. 1999; Serotec, Kidlington, Oxford, UK), Ret (Viglietto et al. 2000; R & D Systems, Abingdon, UK), EE2 (Falcatori et al. 2004; a kind gift from Prof. Yoshitake Nishimune, Japan), or a nuclear protein marker of spermato- gonia Oct-4 (Pesce et al. 1998, Ohbo et al. 2003; Santa Cruz Biotechnology) using dilutions of 1:100, 1:200, 1:20 1:10^4, and 1:100 respectively. In addition, testicular cytospin samples were immunolabeled with antibodies directed against the macrophage marker ED1 (Dijkstra et al. 1985; dilution 1:100; Acris, Hiddenhausen, Germany) and the Sertoli cell marker GATA-4 (Ketola et al. 2002; dilution 1:200; Santa Cruz Biotechnology, kindly supplied by Prof. Markku Heikinheimo, University of Helsinki, Finland). The cytospin samples were then washed twice with PBS and incubated with biotinylated horse anti-mouse, horse anti-goat, or goat anti-rat IgG antibodies (all purchased from Vector Laboratories, Burlingame, CA, USA) at dilutions of 1:250, 1:300, and 1:500 respectively for 30 min at RT. After two more washes with PBS, the slides were incubated with the ABC reagent (ABC kit, Vector Laboratories) for 30 min and, finally, with a solution of 3,3′-diaminobenzidine (Vector Laboratories) for 0.5–1 min until color developed, followed by counterstaining with hematoxylin (Zymed Laboratories, San Francisco, CA, USA) and mounting with an appropriate medium (Vector Laboratories).

Staining of testicular cytospin samples for the myoid cell marker, alkaline phosphatase, was carried out as described by Palombi & Di Carlo (1988). 3β-Hydroxysteroid-dehydroge- nase, which is expressed solely by Leydig cells, was detected by incubating the cytospin slides with a solution containing 2.5 mg β-NAD, 0.6 mg NBT, and 1.5 mg etiocholan-3β-ol-17-one per milliliter of methanol at 37°C for 1 h, followed by two washes with PBS and fixation in 10% formalin (Payne et al. 1980). Under a light microscope, 500 cells on each slide were examined for positive immunostaining. Negative controls were prepared either by omitting the primary antibody or by incubating the slides with irrelevant primary antibodies.

**Labeling of cells with PKH dyes**

In accordance with the manufacturer’s instructions, 4.0 × 10^6 testicular cells isolated from healthy 40-day-old PVG rats or the same number of cells from the lymph nodes of leukemic rats were labeled with PKH 26 or PKH 67 (Sigma) respectively. Following dilution to obtain 20 × 10^6 labeled cells/ml medium, the testicular and leukemic cells were mixed at ratios of 100:1 or 10:1 and these mixtures subsequently incubated for 1, 2, or 3 h. Thereafter, four smears prepared from each such mixture and at each time point were analyzed under a fluorescence microscope (Nikon Eclipse 800; Tokyo, Japan), with leukemic cells emitting green fluorescence (analyzed using a FITC filter) and germ cells red fluorescence (a CY-3 filter). All image processing was performed utilizing the Image-J software (NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/).

In addition, testicular cells from leukemic rats were also labeled with mouse anti-rat Ep-CAM^{−Alexa}, CD4^{−PE}, and MHC Cl I^{−PE} antibodies at the same concentrations and as described above. Subsequently, aggregation of leukemic and germ cells was analyzed by FACS by gating for FSC^{−high} and SSC^{−low}
(Fig. 1G gate R1) and on the basis of the ratio of expression of CD4 and MHC Cl I versus that of Ep-CAM (as the setting for FACS sorting; Fig. 1H).

**Enzymatic digestion of leukemic lymphoblasts**

After separation on Ficoll, peripheral blood lymphoblasts from leukemic rats were either maintained in a medium at 34°C; subjected to enzymatic digestion employing a procedure that has been used to obtain single-cell suspension from testicular tissue (Jahnukainen et al. 2001); or subjected to this same procedure with the omission of the enzyme. Thereafter, the cells from all three groups were labeled separately with mouse anti-rat CD4, CD8, CD45, CD90 and MHC Cl I antibodies as described above, and FACS analysis performed.

**Statistical analyses**

The quantitative data in the figures and tables are presented as mean values ± S.E.M. The one-way ANOVA and t-tests were employed for statistical comparison of independent groups of samples, with a P value of <0.05 being considered to indicate a significant difference.

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- The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

**References**


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