Intratesticular Transplantation of Testicular Cells from Leukemic Rats Causes Transmission of Leukemia

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ABSTRACT

A rat T-cell leukemia model was used to study the safety of germ cell transplantation as a mean of preventing infertility in males undergoing gonadotoxic cancer treatment. Donor germ cells were harvested from the testes of terminally ill leukemic rats and were either used directly or cryopreserved and thawed before transplantation by rete testis microinjection. All rats transplanted with testicular cells from leukemic donors developed signs of terminal rat T-cell leukemia, whereas control animals remained healthy. Cryopreservation of the donor germ cells caused a 3- to 6-day delay in the terminal phase of leukemia. When a known number of leukemic cells were mixed with germ cells and microinjected into the testis, the rate of appearance of terminal leukemia was directly related to the number of transferred leukemic lymphoblasts. As few as 20 leukemic cells were able to cause a cancer relapse resulting in terminal leukemia 21 days after transplantation in three of five transplanted animals. Our results demonstrate that germ cell transplantation with the presently used techniques is not safe enough for clinical use. Improved methods for purging testicular specimens of cancer cells or totally new approaches with transient xenogenetic host models to detect contamination of malignant cells must be developed before this technique can be offered to patients without fear of disease relapse.

INTRODUCTION

There are numerous clinical reports describing adverse effects of cytostatic agents on future fertility of childhood cancer patients (1–4). Severe testicular damage has been observed among patients who have received treatment according to the LSA protocol (5) and after chemotherapy with alkylating agents (6). The pretreatment conditioning for bone marrow transplantation including cytostatic drugs and total body irradiation causes gonadal damage and infertility (7). Major intensification of therapy, which has led to better survival during recent decades, will most probably increase the gonadal damage and infertility in the future (8).

No clinically useful methods for protecting spermatogenic cells from chemotherapy-induced damage have been introduced thus far. Endocrine therapy with the aim of turning off testicular activity by using agonists or antagonists to gonadotrophin-releasing hormone does not seem to be clinically useful as a protective means (9, 10). Most pediatric cancer patients are too young for pretreatment sperm banking, and poor semen quality attributable to malnutrition and the cancer disease itself may decrease the clinical application of this method even among pubertal boys (11).

Transplantation of germ cells to mouse testes made sterile by cytostatic drug treatment has been reported by Brinster and Zimmermann (12) and, recently, successful germ cell transplantation to pri-

mate testes has also been achieved (13). This technique may provide new potential tools for treatment or prevention of infertility in human males. The germ cells may also be frozen for extended periods before transplantation (14). The observation that spermatogenesis can be initiated from stem cells recovered from prepubertal testes (15) suggests that the application of this method may be extended even to pediatric cancer patients. It might be possible that the cryopreserved cells collected from testes of cancer patients before the sterilizing treatment could insure the gene bank of the cancer patient (16).

However, there is no knowledge of possible limitations of spermatogonial transplantation in clinical use. For instance, it is not clear whether germ cells from cancer patients are adequate for harvest or will tolerate cryopreservation. Cancer itself can have an effect on the testis and its ability to support the transplanted germ cells (17). A great concern is also the obvious risk of retransplanting contaminating cancer cells with stored autologous germ cells into a cured patient. This aspect was explored in the present study, undertaken to evaluate the risk of transmission of leukemia during transplantation of testicular cells from leukemic rats.

MATERIALS AND METHODS

Host Animals. Male piebald variegated (PVG) rats at the age of 25 days were treated with one dose of busulfan (10 mg/kg) administered i.p. to destroy the stem spermatogonia in the seminiferous tubules. Animals were maintained in the institute animal house under standard conditions. Animal ethics committee approval for all experiments was given by the Northern Stockholm Animal Ethics Committee No. N169/97.

Preparation of Donor Animals with Rat T-cell Leukemia. The rat T-cell leukemia cell line has been isolated from spontaneously leukemic rats of the Oxford hooded (syngeneic with piebald variegated/c, PVG) inbred strain. The leukemic cells have been shown to express the rat T-cell marker Frc receptor (18). The leukemia is maintained by serial i.p. passage every 17th day (19).

Leukemic rats were killed by CO2 inhalation when approaching the terminal phase (characterized by continuous piloerection, weight loss, and cervical lymph node enlargement; Ref. 18). A leukemic cell suspension (concentration: 1 × 107 cell/ml) was prepared from five enlarged cervical lymph nodes as described earlier (19). Transmission was made by i.p. inoculation with 1000 cells/g body weight to three rats aged 25 days. Age-matched controls received saline. When the signs of spreading leukemia were seen 14 days after inoculation (19), treated animals and controls were sacrificed for donor cell harvest (leukemic donors: body weights, 75 ± 13 g; spleen weights, 11 ± 0.1 g; control donors: body weights, 96 ± 4 g; spleen weights, 0.3 ± 0.0 g).

Harvest of Donor Germ Cells. One-half of one testis from each leukemic and control donor was carefully separated with a knife and immersion-fixed in Bouin’s fixative. The samples were embedded in paraffin and prepared for light-microscopic examination of testicular morphology. The other half and the second testis of the same animal was decapsulated, minced into smaller pieces in Eagle’s MEM with Eagle’s salts (Life Technologies, Inc., Paisley, United Kingdom) supplemented with sodium bicarbonate (15 mM), glutamine (4 mM), HEPES (15 mM), nonessential amino acids, penicillin (100 units/ml), and gentamicin (40 μg/ml). This medium (MEM) was used for all procedures during cell preparation, injection, and freezing. Testis tissue was placed in medium containing 0.5 mg/ml collagenase/dispace (digestion I; Boehringer Mannheim Biochemicals, Mannheim, Germany) and 0.03 mg/ml DNase (Boehringer Mannheim Biochemicals) and incubated in a shaking water bath at 32°C for 10 min to separate interstitial cells. After centrifugation at low
speed (16 × g, 2 min) the supernatant, containing the cells from the interstitium, was discarded except in experiment 3. After removing interstitial cells, a second 15-min digestion (digestion II) with the same enzymes was performed, and the dispersed tubules, pelleted by centrifugation (16 × g, 2 min), were further digested (digestion III) for 20 min at 32°C with 0.5 units/ml heparinase III (Sigma), 0.5 mg/ml collagenase type IV (Sigma), 0.3 units/ml chondroitinase ABC (Sigma), and 0.03 mg/ml DNase. After centrifugation (16 × g, 2 min), the resulting supernatant was pooled with the supernatant testicular cell suspension obtained by digestion II and centrifuged at 200 × g for 10 min. Finally, the cells in the pellet were suspended in MEM to the volume used for microinjection.

In experiments 1 and 2, freshly isolated tubular cells from one leukemic and one control donor in each experiment were used, and in experiment 3, cryopreserved tubular cells from the same leukemic donors and cryopreserved tubular and interstitial cells from the third leukemic donor were used. The number of donor cells injected per testis are indicated in Table 1. Enzymatic digestion of one-and-a-half testis in experiment 1 gave 25 × 10⁶ cells from the control donor and 25 × 10⁶ cells from the leukemic donor and in experiment 2, 20 × 10⁶ and 25 × 10⁶ cells, respectively. Observation of the cell suspensions in a phase-contrast microscope showed testicular single cells with normal morphology. Sertoli cells and cells from spermatocyte and spermatid suspensions in a phase-contrast microscope showed testicular single cells with normal morphology. Sertoli cells and cells from spermatocyte and spermatid suspensions in a phase-contrast microscope showed testicular single cells with normal morphology. Sertoli cells and cells from spermatocyte and spermatid suspensions in a phase-contrast microscope showed testicular single cells with normal morphology.

Cryopreservation of Donor Germ Cells. For cryopreservation, an equal volume of freezing medium (FCS, MEM, and DMSO in a ratio of 1:3:1) was added slowly by drop to the original fresh donor cell suspension and mixed (14). The diluted suspension was dispersed into freezing vials (1 ml/vial), which were placed in an insulated container at ~70°C for 53–111 days. Before transplantation in experiment 3, the cells were thawed at 37°C in a water bath, washed in MEM, and suspended to a volume of 300 μl. The viability of the cells judged by trypan blue exclusion had decreased markedly, and from morphological appearance, round and elongating spermatids were the main group of the dead cells. The overall survival rate of cells after cryopreservation and thawing ranged between 3–16%.

Germ Cell Transplantation. At the age of 45 days, 20 days after the busulphan treatment, the recipient animals were anesthetized with sodium pentobarbitone (50 mg/kg). Using sterile techniques, a caudal midline abdominal incision was made and the testes exposed. The rete of the right testis was localized, punctured, and injected in a retrograde direction under direct vision using a 30-gauge insulin needle connected to polyvinyl chloride tubing and a peristaltic pump. Using the peristaltic pump, the tubing and needle were prefilled with the testicular suspension. Five μl of trypan blue (0.4 mg/ml) were finally added to the tubing and used as an injection marker. The injection volume was 30 μl in experiments 1, 2, and 4 and 60 μl in experiment 3. The number of recipient animals are indicated in Table 1 and in Fig. 3. The injection was confirmed as intratubular when dye was observed in the seminiferous tubules (Fig. 1) and the cell suspension was seen to move inside the tubular compartment during pumping. It was very obvious when the injection was made in the rete and tubules, and when it went into the interstitium. Earlier studies have shown that colloidal carbon successfully injected into the rete in this way is found in the tubules, the rete testis, and the epididymis, suggesting that leakage into the interstitium is minimal. The number of intratubular and interstitial injections are indicated in Table 1.

Assessment of Spreading of the Leukemia after Testicular Microinjection. Signs of leukemia were evaluated daily after microinjection and when the leukemic rats were approaching the terminal phase they were killed by carbon dioxide inhalation. The rats injected with testicular samples from healthy donors showed no leukemic signs but were sacrificed at the same time as the leukemic rats to act as controls. Body, testis, spleen, and right seminal vesicle weights were measured, and the testes were fixed in Bouin’s fixative. The samples were embedded in paraffin and prepared for light-microscopic examination of testicular morphology.

### RESULTS

#### Testicular Morphology of Leukemic and Healthy Donors. Histological sections of leukemic donor testes showed blood vessels filled with leukemic cells. Some interstitial infiltrates of leukemic cells were also seen (Fig. 2A). The morphology of control testes was normal (Fig. 2B).

#### Transmission of Leukemia after Spermatogonial Transplantation. Fourteen days after intratubular or interstitial injection, all hosts receiving fresh testicular cells from a leukemic donor developed typical signs of terminal rat T-cell leukemia, whereas control animals remained healthy (Table 1). Injection with cryopreserved germ cells caused a 3- to 6-day delay in the terminal phase of leukemia (Table 1).
with a shorter interval in the animal group that received cells from the sample showing the best viability after thawing. The terminal phase was reached after 17 days also in the animal group that received cells from the interstitial tissue preparation. All leukemic animals showed very similar spleen enlargements compared with controls (Table 1), and they also had smaller seminal vesicular glands (Table 1), which is a good indicator of lower androgen levels. The injection site in either the intratubular compartment or the interstitial compartment had no effect on terminal phase timing or on terminal body weights (Table 1).

Testicular Morphology after Transplantation of Freshly Isolated Donor Cells. Testes of the animals microinjected with freshly isolated cells from leukemic donors were marked enlarged compared with contralateral control testes or the testes of the animals injected with healthy donor cells (Table 1). No statistically significant difference was seen in the size of the testes after intratubular or interstitial injection of fresh cells from leukemic donors (Table 1), and the injection site of cells from leukemic donors did not affect the histological morphology. The interstitial tissue was filled with leukemic cells, and malignant lymphocytes with active cell division were seen (Fig. 2C). The seminiferous tubules showed degenerating germ cells, and occasionally empty tubules without Sertoli or germ cells were seen (Fig. 2C). The contralateral untouched testis showed Sertoli cells-only tubules with and without degenerating spermatogenetic cells as a result of the busulfan treatment. This histological picture was very similar to that in microinjected (Fig. 2D) and uninjectected testes of control animals. However, in the uninjected testes of the leukemic recipient animals, some few leukemic infiltrates were seen in the interstitium just beneath the capsule.

Testicular Morphology after Transplantation of Cryopreserved Donor Cells. After transplantation of cryopreserved testicular cells from leukemic donors, testicular enlargement appeared more slowly. The testicular size was significantly ($P < 0.05$) smaller after intratubular injection (0.92 ± 0.51 g) than after interstitial injection (1.62 ± 0.17 g) of cryopreserved cells in experiment 3. Interstitial injections caused marked enlargement of the testes (Table 1) and destruction of the morphology of testicular tubules by dividing leukemic cells to an extent comparable with the finding after intratubular injection with freshly isolated testicular cells (Fig. 2C). Intratubular injection with cryopreserved cells caused a tumor-like infiltration of the caput epididymidis, which adhered firmly to the testicular capsule, in 6 of 12 cases. In these cases, testes remained small and atrophic (Table 1). In the histological examination, the testes showed empty seminiferous tubules, and small leukemic infiltrates were seen in the interstitial tissue. The contralateral uninjectected testes had a morphology and size comparable with the controls (Table 1).

Number of Transplanted Leukemic Cells and Host Survival. All hosts receiving an intratubular injection of 6000 and 200 leukemic cells mixed with 1.5 × 10⁶ testicular cells from healthy donors developed terminal leukemia in 14 and 19 days, respectively. In the group injected with 20 leukemic lymphoblasts per testis, three of five animals showed signs of terminal leukemia 21 days after transplantation (Fig. 3). The other two animals and all six animals receiving only two leukemic cells remained healthy and kept on growing for the entire observation time of 42 days. No signs of leukemia were seen at the termination of the experiment in these latter animals.

DISCUSSION

This study shows that germ cell transplantation with fresh and frozen testicular cells from leukemic donor rats transmits acute leukemia to healthy recipients. An immediate conclusion from our findings is that spermatogonial transplantation with unpurged testicular samples should not be used for any leukemic patient to restore their fertility. Even patients with lymphoma or metastasising cancers might incur a risk of recurrence, because cancer cells circulating in blood vessels may contaminate testicular biopsies. Our data also shows that as few as 20 leukemic cells are able to cause a relapse of the malignant disease when injected into the testis.

Manipulation of the testicular samples might change the risk of recurrence after spermatogonial transplantation, because cryopreservation of the donor cell population was able to change the aggressive course of the leukemia and the testicular infiltration. Cryopreservation most probably decreased the number of viable leukemic cells in the samples, inasmuch as the speed of appearance of terminal leukemia was directly related to the number of leukemic lymphoblasts injected. The histological morphology of leukemic donor testes suggests that most leukemic cells originate from the interstitial tissue (Fig. 2A). Accordingly, the more aggressive course of the leukemia seen after injection of interstitial cells instead of
tubular cells also supports the finding that the leukemic cell content of the donor sample is related to the timing of terminal leukemia.

The small sample size of the testicular biopsies will present problems in purging the cancer cells from the spermatogenetic stem cells. The cell contents of testicular biopsies would constitute only a small proportion of the cell number transplanted in the present study, and orchiectomy for sample collection would not be acceptable in clinical practice. One possibility to circumvent this problem would be in vitro maintenance of testicular stem cells. Reinitiation of spermatogenesis after culture of spermatogonia for several months has shown to be successful in mice (20). Such in vitro culture of testicular tissue might also deplete malignant cells before autologous retransplantation. Another possibility could be to use xenologous immunodeficient hosts such as severe combined immunodeficiency mice to preassess testicular samples for cancer-cell contamination and engrafment capacity of the stem cells. Xenologous transplantation might be useful also to maintain and grow the stem cell population. The observation that rabbit and dog germ cells colonize the seminiferous tubules of immunodeficient mice, but show no differentiation, suggests that initiation of complete spermatogenesis may occur only between closely related species (21).

The present results show that spermatogenic donor cells can be harvested from terminally ill leukemic animals, although their functional capacity to reconstitute spermatogenesis was not investigated. The cells can be cryopreserved and thawed like spermatogenic cells from healthy donors. This may differ from earlier observations that sperm collection and sperm cryopreservation are difficult after malignant disease (11). In line with this observation is the previous finding that the spreading leukemia disturbs especially the later differentiation steps of spermatogenesis, which involves haploid cell types, whereas more immature germ cell types are preserved (17). However, because of the short observation time and early occurring leukemic symptoms, the ability of stem cells obtained from leukemic donors to initiate spermatogenesis could not be evaluated in the present study.

It is remarkable that when freshly isolated samples were injected intratubularly, the leukemic cells showed the capacity to invade the testicular interstitium. The histological morphology of these testes was comparable with that of interstitially injected testes or to the testicular morphology after intratesticular transplantation with rat leukemic T-cells (19) that was reported previously. This may indicate that a bigger number of more invasive and proliferative leukemic cells...
in freshly isolated testicular samples are able to penetrate the seminiferous tubule wall and cause a direct infiltration into the interstitium.

The present results confirm the earlier observation that the testis has barriers decreasing the leukemic cell infiltration into the interstitium (19, 22). The vascular endothelium of intact testis has previously been shown to restrict the penetration of the leukemic cells from the circulation into the testis (19, 22). The present results support the earlier observations that similar barriers may exist also between the tubular and the interstitial compartment (19, 22).

In conclusion, this study shows that, with the harvest and transplantation techniques used presently by us and others, spermatogonial transplantation is not safe enough for clinical use. Improved methods for the purging of testicular samples of cancer cells and the enrichment of spermatogenetic stem cells, or totally new approaches with passage through xenogenic host models, must be developed before this technique can be offered as a means to rescue, without the fear of disease relapse, the future fertility of male cancer patients.

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