Dose–response of RAG2^{-/-}/γ_c^{-/-} mice to busulfan in preparation for spermatogonial transplantation

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Practical applications of spermatogonial transplantation require good rates of colonization by the donor cells. Recipient testes are usually depleted of competing endogenous spermatogonia by administration of 32–44 mg busulfan kg⁻¹ body weight before transplantation. However, it is not clear that this is the optimum dose, especially for immunodeficient mice. In the present study, the response of adult RAG2^{-/-}/γ_c^{-/-} (RAG2) male mice to treatment with 10–50 mg busulfan kg⁻¹ body weight was determined in terms of mortality rates, testicular masses and histology, and colonization of seminiferous tubules by transplanted spermatogonia. Mortality increased from 0 to 50% at doses between 20 mg busulfan kg⁻¹ and 40 mg busulfan kg⁻¹, whereas the maximum effects on testicular mass and histology were observed at 20 mg busulfan kg⁻¹. Colonization of testes by genetically marked spermatogonia after treatment of mice with 20 mg busulfan kg⁻¹ was

equivalent to rates previously reported in recipients treated with 32–44 mg busulfan kg⁻¹. Thus, 20 mg busulfan kg⁻¹ appears to be the optimum dose for preparing RAG2 mice for spermatogonial transplantation. However, because the steepness of the dose–response curves indicates that direct administration of busulfan is not ideal for this purpose, 15 mg busulfan kg⁻¹ was administered to pregnant females at various times between day 10.5 and day 16.5 of gestation to determine whether this would deplete the number of germ cells in male offspring. Although there were large variations in testicular mass and histology, no mortality was observed and administration of busulfan at day 10.5 or 12.5 after mating delayed initiation of spermatogenesis, indicating that prenatal administration of busulfan combined with neonatal transplantation might be an effective method for further increasing rates of colonization by donor spermatogonia.

Introduction

Spermatogonial transplantation is a valuable technique for studying spermatogenesis and cell–cell interactions in the testis, and is also a potentially powerful tool of assisted reproductive technology (for reviews, see Nagano and Brinster, 1998; Johnston *et al.*, 2000; Russell and Griswold, 2000). Efficient colonization of seminiferous tubules by the transplanted cells is required for spermatogonial transplantation to achieve its potential. Shinohara *et al.* (2001, 2002) suggested that such colonization might depend on how well the transplanted cells compete with the endogenous spermatogonia for the available stem cell niches, and that this depends on the relative numbers of spermatogonia in the two populations and access of the transplanted cells to the basal compartment of the seminiferous tubules.

Several techniques deplete the seminiferous tubules of differentiating germ cells, but few decrease the number

of stem cell spermatogonia. Methods to reduce the number of germ cells in testes have included irradiation (Craig *et al.*, 1958; Oakberg, 1959; Dym and Clermont, 1970; Izadyar *et al.*, 2000), experimental cryptorchism (Shinohara *et al.*, 2000), heat treatment (Jégou *et al.*, 1984), GnRH antagonist treatment (Schlatt *et al.*, 1999), cold ischaemia (Young *et al.*, 1988) and sterilizing drugs (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Clouthier *et al.*, 1996; Ogawa *et al.*, 2000). In addition, a mouse strain that is sterile as a result of a mutation of the c-kit receptor gene (homozygous for the white spotting allele; W/W) has been used in transplantation studies (Russell *et al.*, 1996; Tanemura *et al.*, 1996; Parreira *et al.*, 1998, 1999). Ideally, the cells most affected by treatment should be the type A spermatogonia, and in particular the A_s or A₀ spermatogonia (de Rooij and van Dissel-Emiliani, 1997), which are the stem cells of the testis. Irradiation, cryptorchism, heat treatment and GnRH antagonists primarily decrease the type B spermatogonia or the later stages of spermatogenesis, although fractionated ionizing irradiation reduces the population of stem cell spermatogonia (Izadyar *et al.*, 2000; Creemers *et al.*, 2002). Two other methods that

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result in reduction of A_s spermatogonia have practical disadvantages: cold ischaemia requires surgical intervention before the transplantation surgery, and the genetically sterile mouse strains are not immunodeficient and can be used only for syngeneic transplantations. The common means of preparing recipient mice for spermatogonial transplantation is by treatment with busulfan, a DNA alkylating agent (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Clouthier *et al.*, 1996; Ogawa *et al.*, 1997, 2000). Busulfan targets the slowly proliferating A_s spermatogonia (Hemsworth and Jackson, 1962; Dunn, 1974) and is easily administered by a single i.p. injection. Although impaired function of Sertoli or Leydig cells has not been reported, Brinster and Zimmermann (1994) suggested that busulfan may disrupt junctions between Sertoli cells and thus aid in the movement of transplanted spermatogonia into the basal compartment.

The sterilizing dose of busulfan is species-specific: 6–100 mg busulfan kg⁻¹ body weight (hereafter expressed as mg busulfan kg⁻¹ for brevity) in rodents (Bucci and Meistrich, 1987; Kim *et al.*, 1997), 40–100 mg busulfan kg⁻¹ in pigs (Kim *et al.*, 1997) and 4–12 mg busulfan kg⁻¹ in coyotes (Stellflug *et al.*, 1985). The lowest dose of busulfan that has been administered to adult mice before spermatogonial transplantation is 32 mg busulfan kg⁻¹ in a single i.p. injection (Clouthier *et al.*, 1996), but 40 mg busulfan kg⁻¹ is the dose commonly used for both immunocompetent and immunodeficient mice (Ogawa *et al.*, 1999). Recipient rats have been prepared by two injections of busulfan at the dose of 10 or 15 mg busulfan kg⁻¹ (Jiang and Short, 1995; Jiang, 1998). Dose–response relationships for busulfan have been examined only in immunocompetent mice, and inconsistencies of vehicle and method of evaluation confuse the literature. Furthermore, it is not clear whether dose–response studies in immunocompetent mice are applicable to immunodeficient mice. Adverse effects of busulfan on the animals' health have been mentioned in the spermatogonial transplantation literature, but have not been quantified (Clouthier *et al.*, 1996; Dobrinski *et al.*, 2000).

Therefore, the first aim of the present study was to examine the dose–response relationship for busulfan in RAG2^{-/-}/γC^{-/-} (RAG2) mice that are T-, B- and natural killer (NK)-cell deficient (Colucci *et al.*, 1999). Specifically, an optimum dose of busulfan for depleting adult RAG2 seminiferous tubules of germ cells was determined by assessing testicular mass and morphology over the time period critical for transplantation, and by determining rates of mortality at the various doses. Rates of exogenous spermatogenesis in RAG2 mice prepared with the optimal dose of busulfan were measured for comparison with those previously published.

The second aim of the present study was to examine the feasibility of depleting recipient RAG2 males of germ cells *in utero* by administering a low dose of busulfan to

pregnant females. These experiments were suggested by the results of previous research in rats: Hemsworth and Jackson (1963) reported that male offspring of female rats injected with a single dose of 10 mg busulfan kg⁻¹ between day 13 and day 20 after mating were sterile, on the basis of histological examination; Gaulier and Roux (1970) found an increase in the number of seminiferous tubules devoid of germ cells in the male offspring of female rats injected with 20 mg busulfan kg⁻¹ between day 9 and day 13 after mating, and day 11 after mating was the most effective time for treatment. In the present study, the possibility that a single systemic busulfan dose of 15 mg kg⁻¹ administered to females at any of four time points between day 10.5 and day 16.5 after mating would deplete germ cells in male RAG2 offspring was examined.

Materials and Methods

Animals

RAG2 mice were obtained from the breeding colony of A. Croy (Department of Biomedical Sciences, University of Guelph, Guelph). The mice were maintained in a germ-free isolation facility on campus at 22–25°C under a 10 h light:14 h dark cycle. Food and water were autoclaved and were available *ad libitum*. ROSA26 males (B6,129-GT(ROSA)26) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained and treated in accordance with the guidelines of the Canadian Council of Animal Care.

Administration of busulfan

Adult male dose–response study. Seventy-six male mice aged 6–16 weeks (19–40 g) were randomly allocated to experimental groups consisting of at least three mice. Mice in treatment groups were given a single i.p. injection of 50 (*n*=12), 40 (*n*=12), 30 (*n*=14), 25 (*n*=8), 20 (*n*=9) or 10 (*n*=6) mg busulfan kg⁻¹ or two injections of 10 mg busulfan kg⁻¹ given 7 days apart (*n*=6). Control animals (*n*=9) received a single injection of 0.2 ml of a 1:1 mixture of dimethyl sulphoxide (DMSO) and water, the vehicle. Aliquots of 24.63 mg busulfan ml⁻¹ (ICN Biochemicals, Aurora, OH) in DMSO (Sigma, St Louis, MO) were stored at –80°C. The stock solution was diluted to a final concentration of 1.5–7.5 mg ml⁻¹ busulfan in 50% (v/v) DMSO immediately before use. The mice were weighed and given a single injection of busulfan (0.15–0.35 ml). The health of the animal was assessed by inspection on the day after treatment and then regularly until the testes were collected.

In utero administration. Females were caged with males overnight and mating was assessed by the presence of a vaginal plug on the following morning (day 0.5 after

mating). Pregnant females were injected with a single i.p. dose of 15 mg busulfan kg^{-1} at day 10.5 ($n=13$), 12.5 ($n=15$), 14.5 ($n=11$) or 16.5 ($n=16$) after mating. The male pups from each litter were divided into two groups for testes collection at age 5 or 9 weeks.

Spermatogonial transplantation into busulfan-treated mice

Preparation of recipient males and controls. In view of the results from the dose-response study, adult male RAG2 mice aged 11–28 weeks ($n=14$; 22–35 g) were given a single i.p. injection of 20 mg busulfan kg^{-1} approximately 4 weeks before spermatogonial transplantation. As injected controls, two busulfan-treated RAG2 mice received only medium containing 0.032% (w/v) Trypan blue (see below). In addition, for each transfer, only one testis was injected, and the other testis served as a contralateral uninjected control.

Preparation of donor cells. The testicular cells from six adult male ROSA26 mice transgenic for a gene encoding a bacterial β -galactosidase were pooled and processed as described by Bellvé *et al.* (1977) with minor modifications. In brief, the testes were rinsed in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies, Burlington, ON) and the tunicae were removed with forceps. The testes were bisected and incubated in 10 ml of 0.1 mg collagenase ml^{-1} (Type IV; Sigma) in enriched Krebs–Ringer bicarbonate (EKRb) at 38.5°C in an atmosphere of 5% CO_2 in air for 15 min with gentle shaking. The collagenase solution was decanted; the tissue was washed three times in EKRb, and further digested in 5 ml of 1 mg trypsin ml^{-1} and 1 μg DNase I ml^{-1} (Sigma) in EKRb for 20 min under the same conditions. The enzyme solution was decanted and the cells were resuspended in 10 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 1% (v/v) penicillin–streptomycin (Life Technologies) for filtration through a PeCapp polyester monofilament screen (opening = 52 μm ; B & SH Thompson Co. Ltd, Toronto, ON) to remove debris. The result was a crude suspension of ROSA26 spermatogonia including all stages of germ cells as well as some somatic cells. Donor spermatogonial cells were frozen in the above medium containing 10% (v/v) DMSO. Just before injection, the cells were thawed, washed and resuspended in DMEM containing 1% (v/v) penicillin–streptomycin, 0.05% (v/v) MEM amino acids and non-essential amino acids (Life Technologies), 110 μg sodium pyruvate ml^{-1} , 0.1% (w/v) polyvinylalcohol and 0.032% (w/v) Trypan blue (Sigma).

Microinjections. The transplantations were performed aseptically within a laminar flow hood. The injection apparatus and the injection technique were basically as described by Ogawa *et al.* (1997). Recipient mice were

anaesthetized with Avertin by i.p. injection. Avertin is a mixture of 2,2,2-tribromoethyl alcohol and tert-amyl alcohol (Aldrich Chemical Co., Milwaukee, WI) and was prepared and used according to Hogan *et al.* (1994). One testis was exteriorized and the efferent duct was dissected free of surrounding fat. Tension on the duct was created by placing a pair of microdissection forceps with slide (Fine Science Tools, Vancouver) under the duct. The efferent duct was cannulated and the micropipette was threaded to the junction of the efferent duct with the rete testis. The donor cell suspension (3×10^6 – 2×10^7 cells ml^{-1} , 11–15 μl per testis) was injected by manually increasing the pressure in the micropipette through a 10 ml syringe. When approximately 80% of surface seminiferous tubules were seen to be filled with the blue suspension, the pipette was removed and the body wall was closed. An analgesic, buprenorphine (0.1 mg kg^{-1} , Buprenex; Colman Pharmaceutical Inc., Richmond, VA), was administered i.p. as soon as the animals began to wake. The recipients were returned to the isolation facility until autopsy was performed.

Processing and analysis

Busulfan studies. Testes of adult males were collected at weeks 4 and 10 after administration of busulfan. The testes of male offspring of mice treated during pregnancy were collected at weeks 5 and 9 after birth. For each individual, both testes were dissected free from the epididymis and surrounding fat and weighed (± 0.0001 g). Subsequently, one testis from each animal was fixed overnight in Bouin's fixative and the other in 4% (w/v) paraformaldehyde in PBS. Both testes were embedded in paraffin wax using standard procedures, and cut into serial sections at 5 μm thickness up to the approximate mid-line. Three non-adjacent sections per half testis were selected about 25 sections apart, mounted on slides and stained with haematoxylin–eosin. The slides were dehydrated through ethanol and xylene before mounting in Permount (Fisher Scientific, Nepean).

Testicular masses were compared using simple ANOVA (SAS, 6.12). The experimental unit was the mouse and therefore the mean of the two testicular masses was used. The results were transformed to a logarithmic scale to normalize the data. Differences were considered significant if a one-tailed test had $P \leq 0.05$. For each individual testis, three non-adjacent sections were examined blind by three observers. Approximately 20 tubules were scored per section, and each seminiferous epithelium was classified as having: no spermatogenesis (no evidence of regeneration of a seminiferous epithelium); partial spermatogenesis (spermatogonial proliferation and spermatocytes but no later stages of spermatogenesis); or full spermatogenesis (a complete seminiferous epithelium with spermatids and spermatozoa). The incidence of these categories was compared using blocking ANOVA (SAS, 6.12). The results were

Fig. 1. Estimated probability of no spermatogenesis in a cross-section of a seminiferous tubule (◆), rates of mortality (■) and mean testicular masses (▲) of adult RAG2 mice at (a) week 4 and (b) week 10 after administration of 0–50 mg busulfan kg⁻¹ body weight. The control groups (0 mg busulfan kg⁻¹, $n=5$ at week 4 and $n=4$ at week 10) were injected with vehicle only. The number of animals (two testes per animal) analysed to determine the amount of spermatogenesis and testicular mass in each treatment group were as follows: 10 mg busulfan kg⁻¹, $n=3$ at week 4 and $n=3$ at week 10; 20 mg busulfan kg⁻¹, $n=6$ at week 4 and $n=3$ at week 10; 25 mg busulfan kg⁻¹, $n=4$ at week 4 and $n=3$ at week 10; 30 mg busulfan kg⁻¹, $n=3$ at week 4 and $n=6$ at week 10; 50 mg busulfan kg⁻¹, $n=4$ at week 4 and $n=3$ at week 10. The differences in the number of animals analysed for spermatogenesis and testicular mass and number to which busulfan was administered are due to mortality. Mean masses were compared using a nested ANOVA after log transformation to normalize the data. The probabilities of no spermatogenesis were compared using blocking ANOVA and the results were transformed with a logit scale to normalize the data. In both cases, error bars indicate 95% confidence intervals.

transformed using a logit scale to normalize data. Testes from animals that died during treatment were not included in the analyses.

Transplantation recipients. Testes from recipients of ROSA26 spermatogonia were collected at day 5 ($n=3$), and weeks 4 ($n=4$), 8 ($n=4$) and 12 ($n=3$) after transplantation, and processed for cryosectioning. In brief, the testes were placed into 4% (w/v) paraformaldehyde in PBS on ice overnight. The testes were equilibrated first in 15% (w/v) sucrose (Sigma) and then in 30% (w/v) sucrose in PBS, placed in optimal cutting temperature compound (Tissue-Tek, Torrance, CA), plunged into liquid nitrogen and stored at -80°C . Sagittal sections (5 μm thickness) were cut from half of each testis, and the sections were mounted on slides, air dried and stored at -20°C . As the donor cells contained the bacterial *lacZ* transgene, the sections were stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; Boehringer Mannheim, Temicula, CA), a β -galactosidase substrate that forms a blue precipitate on reaction. The protocol was as described in Sanes *et al.* (1986). The sections were counterstained for 1 min in 0.1% (w/v) nuclear fast red (Sigma) in 70 mmol aluminum sulphate l⁻¹, dehydrated through ethanol and xylene, and mounted in Permount. A few sections were also stained with haematoxylin–eosin.

Evidence of repopulation and spermatogenesis from transplanted cells was quantified by counting the number of tubules with β -galactosidase-positive cells in a recipient testis, and by classifying the extent of regeneration by the donor (ROSA26) cells as early (occasional cells or small groups of cells), intermediate (small clusters of spermatogonia or spermatocytes), late (spermatocytes and spermatids) or full (presence of spermatozoa). Furthermore, the overall level of spermatogenesis from the combined endogenous and transplanted cells was quantified in haematoxylin–eosin-stained sections as in the busulfan dose–response experiments.

Results

Busulfan dose–response in adult males

At week 4, the mean testicular mass of mice in each treatment group was lower than in the control group ($P < 0.05$) (Fig. 1a). There were no differences in testicular masses between groups treated with 20–50 mg busulfan kg⁻¹. The 10 mg busulfan kg⁻¹ group had an intermediate mean mass, as did the two doses of 10 mg busulfan kg⁻¹ group (data not shown). At week 10 after

Fig. 2. Representative photomicrographs of the testes of adult male RAG2 mice after administration of various doses of busulfan. (a,b) Testis from a mouse at week 4 after treatment with 20 mg busulfan kg⁻¹ body weight showing tubules classified as having no spermatogenesis. (b) A higher power image of the germ cell-depleted tubule marked with an asterisk in (a). (c,d) Testis of a mouse at week 4 after treatment with two doses of 10 mg busulfan kg⁻¹ showing tubules classified as having partial spermatogenesis. (d) A higher power image of the tubule marked with an asterisk in (c); spermatogonia and spermatocytes are present, but no later stages of spermatogenesis are visible. (e,f) Testis at week 10 after administration of 10 mg busulfan kg⁻¹ illustrating tubules with full spermatogenesis. The tubule marked by an asterisk in (e) is shown at higher power in (f). Sections were stained with haematoxylin–eosin. Scale bar represents (a,c,e) 311 and (b,d,f) 75 μm .

Fig. 2. For legend see facing page.

injection (Fig. 1b), the testicular masses of mice in all treatment groups were still lower than in the controls ($P \leq 0.05$). Again, the 10 mg busulfan kg^{-1} group had an intermediate mean mass, as did the two doses of 10 mg busulfan kg^{-1} group (data not shown).

From the histological examination, the estimated probability of a seminiferous tubule having no spermatogenesis (no seminiferous epithelium) at week 4 was higher in the 20–50 mg busulfan kg^{-1} treatment groups than in the controls ($P \leq 0.05$; Fig. 1a). The difference between the control group and the 10 mg busulfan kg^{-1} group was not significant. Doses of 20–50 mg busulfan kg^{-1} resulted in no spermatogenesis in $> 90\%$ of tubule cross-sections. The 40 mg busulfan kg^{-1} treatment group was omitted from the analyses due to the small number of surviving individuals and high variability in the results. Histological analysis of testes at week 10 (Fig. 1b) indicated that mice in the 20–50 mg busulfan kg^{-1} groups had more tubules with no spermatogenesis than did the controls ($P \leq 0.05$), although the mean number of tubule cross-sections with no spermatogenesis now ranged from 65% to 95%. The probability of partial spermatogenesis was higher than in the control in the 10 mg busulfan kg^{-1} group at week 4 only, and there were no differences in numbers of tubules with partial spermatogenesis in any group at week 10 compared with controls (data not shown). Mean testicular mass and the probability of no spermatogenesis after administration of two doses of 10 mg busulfan kg^{-1} were 0.038 g and 0.659, respectively at week 4, and 0.058 g and 0.070, respectively at week 10 (data not shown). For comparison, the respective values were 0.034 g and 0.961 at week 4, and 0.031 g and 0.830 at week 10 in the 20 mg busulfan kg^{-1} group. In each case, two administrations of 10 mg busulfan kg^{-1} were less effective than a single dose of 20 mg busulfan kg^{-1} ($P \leq 0.05$). The probability of partial spermatogenesis after two doses of 10 mg busulfan kg^{-1} was higher than in controls at week 4 only. On average, busulfan at 20 mg busulfan kg^{-1} was as effective in decreasing testicular mass and depleting the seminiferous tubules of germ cells as any of the higher doses over the 6 weeks studied. Representative sections of testes at weeks 4 and 10 are shown (Fig. 2).

There were no deaths or signs of physical distress in any of the 30 animals given doses of < 25 mg busulfan

kg^{-1} or control vehicle (Fig. 1a). At 25 mg busulfan kg^{-1} , one of eight (12%) mice injected died. At 30 mg busulfan kg^{-1} , five of 14 (37%) mice died, and mortality was approximately 50% in the 40 and 50 mg busulfan kg^{-1} groups (6/12 and 5/12, respectively). Signs of distress were a hunched back, ruffled hair and decreased activity at weeks 2–3 after injection. All mice that exhibited these ill effects died within a day, or, as the study progressed, were immediately killed.

ROSA26 spermatogonial transfers

From the results described above, 20 mg busulfan kg^{-1} was deemed the optimal dose for preparing RAG2 mice for spermatogonial transplantation. Therefore, males treated with 20 mg busulfan kg^{-1} were used as spermatogonial recipients to ensure that they would support exogenous spermatogenesis at a level similar to that reported with animals treated with higher doses. No donor cells expressing β -galactosidase were detected at day 5 after transfer. However, at all subsequent time points (weeks 4, 8 and 12 after transfer), 50–75% of recipients were found to contain cells expressing the transgene using a histochemical assay (Figs 3 and 4), indicating that ROSA26 spermatogonia were successfully transplanted into the seminiferous tubules in most cases. The mean number of tubules per section with cells expressing β -galactosidase increased from 7.5 at week 4 after transplantation (sum of all categories of regeneration in Fig. 4) to 10.0 at week 8 and 12.5 at week 12, indicating expansion of ROSA26 spermatogonia down the seminiferous tubules and hence proliferation of donor spermatogonia. There was also differentiation of the transplanted cells, and the mean numbers of ROSA26 tubules containing spermatids and spermatozoa increased with time. At week 4 after transfer, 27% of tubules with ROSA26 cells were categorized as having intermediate regeneration of spermatogenesis (ROSA26 spermatocytes present), but none showed signs of further differentiation. The percentage of ROSA26 tubules containing late regeneration (spermatids) was 20% at week 8 and 28% at week 12, and the percentage of tubules showing full regeneration (ROSA-derived spermatozoa present) was 5% at week 8 and 16% at week 12.

There was no evidence of bacterial β -galactosidase activity in the injected control testes (injected with

Fig. 3. Photomicrographs of recipient testes of busulfan-treated RAG2 mice at weeks 4, 8 and 12 after transplantation of ROSA26 spermatogonia, and of a negative control testis and a positive control kidney. (a) Negative control: testis of a RAG2 mouse with no endogenous β -galactosidase activity. (b) Positive control: ROSA26 mouse kidney with β -galactosidase activity indicated by blue precipitate. (c) Section of a RAG2 mouse seminiferous tubule at week 4 after transplantation with a few clusters of ROSA26 cells (blue) (early regeneration). (d) Proliferation of ROSA26 cells at week 8 after transplantation (late regeneration). (e) Proliferation of ROSA26 cells and production of donor-derived spermatozoa (arrows) at week 12 after transfer (full regeneration). (f) Low magnification of a recipient testis at week 12 after transfer of ROSA26 cells showing a number of repopulated seminiferous tubule cross-sections. Staining of the interstitial cells in this section is due to the endogenous β -galactosidase, a background activity that occurred in some sections after longer incubations in substrate. Scale bar represents (a–e) 75 and (f) 656 μm .

Fig. 3. For legend see facing page.

Fig. 4. Testicular regeneration at various times after transfer. Mean number of seminiferous tubule cross-sections with different categories of regeneration by ROSA26 cells after transplantation into RAG2 mice. Means were calculated using only the testes that contained β -galactosidase activity (that is, were colonized by donor cells). Degree of regeneration: early (occasional cells or small groups of cells; $\square$); middle (small clusters of spermatogonia or spermatocytes; $\blacksquare$); late (spermatocytes and spermatids; $\square$); and full (presence of spermatozoa; $\blacksquare$). n =the number of recipients with testes that were colonized by ROSA26 cells over the total number of recipients ($n=0/3$ at 5 days; $n=2/4$ at 4 weeks; $n=3/4$ at 8 weeks; and $n=2/3$ at 12 weeks). At day 5 after transplantation, none of the three mice injected with ROSA26 cells contained β -galactosidase-positive cells.

medium and Trypan blue) or uninjected contralateral testes. In addition, when Trypan blue-injected testes were compared with their contralateral controls, no difference in the amount of regeneration from remaining endogenous spermatogonia was observed, indicating that Trypan blue and the injection procedure had little effect on spermatogenesis (data not shown).

Males treated with busulfan *in utero*

With one exception (day 14.5 after mating, week 5), the mean testicular masses of the offspring of females that were injected with 15 mg busulfan kg^{-1} between day 10.5 and day 16.5 after mating were significantly lower than those of their time-paired controls at both week 5 and week 9 ($P \leq 0.05$; Fig. 5). From the histological examination, the estimated probability of there being no spermatogenesis in a tubule cross-section at week 5 after birth was higher in males exposed to busulfan *in utero* at days 10.5 or 12.5 after mating than in the controls ($P \leq 0.05$; Fig. 6). There was no difference at week 5 when the males were exposed at day 14.5 or 16.5 after

Fig. 5. Testicular mass after *in utero* administration of busulfan. Mean testicular masses of the offspring of RAG2 mice that were injected with 15 mg busulfan kg^{-1} ($\blacksquare$) or vehicle alone (0 mg busulfan kg^{-1} ; $\square$) at day 10.5, 12.5, 14.5 or 16.5 of pregnancy. Day after mating on which females were injected with busulfan is shown at the top of the graph. Testicular masses were measured at week 5 or 9 after birth. Error bars indicate the 95% confidence interval (nested ANOVA using log-transformed testicular masses to normalize the data). Numbers above data bars indicate number of individuals analysed (two testes per individual).

Fig. 6. Inhibition of spermatogenesis by *in utero* administration of busulfan. Estimated probability of a cross-section of a seminiferous tubule displaying no spermatogenesis in male offspring of RAG2 females that received 15 mg busulfan kg^{-1} ($\blacksquare$) or vehicle alone (0 mg busulfan kg^{-1} ; $\square$) at day 10.5, 12.5, 14.5 or 16.5 of pregnancy. Day after mating on which females were injected with busulfan is shown at the top of the graph. Numbers of animals in each group are as shown in Fig. 5. Testes were collected for histological examination at week 5 or 9 after birth. Error bars indicate the 95% confidence interval (blocking ANOVA with data normalized using a logit scale). *Indicates that the mean number of tubules with no spermatogenesis is higher than in the control group ($P \leq 0.05$).

Fig. 7. Photomicrographs of the testes of offspring of busulfan-treated female RAG2 mice at week 9 after birth. (a,b) Control testis of an offspring whose dam was injected with vehicle (0 mg busulfan kg^{-1}) at day 10.5 after mating showing a full seminiferous epithelium. (c,d) Testis of offspring of a dam injected with 15 mg busulfan kg^{-1} on day 10.5 after mating showing several depleted tubules. (e,f) Testis of offspring of a dam injected with 15 mg busulfan kg^{-1} on day 12.5 after mating showing many tubules containing only Sertoli cells. Sections were stained with haematoxylin–eosin. Scale bar represents (a,c,e) 311 μm and (b,d,f) 75 μm .

Fig. 7. For legend see facing page.

mating and there was no significant difference in any treatment group by week 9 after birth. These data indicate that the onset of spermatogenesis may be delayed in the males exposed to busulfan at day 10.5 or 12.5 after mating. The probability of partial spermatogenesis at week 5 after birth was higher in the day 12.5 and day 16.5 treatment groups than in controls ($P \leq 0.05$), but at week 9 after birth there was no difference in partial spermatogenesis (data not shown). Representative sections of testes at weeks 5 and 9 after birth are shown (Fig. 7).

Discussion

The principal findings of this study are that 20 mg busulfan kg^{-1} body weight is the appropriate dose of busulfan to prepare RAG2 mice for spermatogonial transplantation, and that busulfan is not an ideal drug for depleting adult testes of germ cells. The results also indicate that further exploration of the treatment of pregnant females with busulfan as a means of obtaining germ-cell-depleted recipients may be warranted.

Doses of between 20 mg busulfan kg^{-1} and 50 mg busulfan kg^{-1} cause equivalent reductions in testicular mass of adult RAG2 mice and in numbers of endogenous germ cells, but doses > 30 mg busulfan kg^{-1} resulted in death of approximately half of the RAG2 mice. This finding was unexpected, because the dose generally administered to Nude and SCID mice before transplantation is a single injection of 32–44 mg busulfan kg^{-1} . The RAG2 mice are T-, B- and NK-cell deficient and have a different genetic background to Nude (T-cell deficient) and SCID (T- and B-cell deficient) mice.

The effects of busulfan on the seminiferous epithelium were measured at weeks 4 and 10 after administration because previous research had demonstrated that it is not until approximately 4 weeks after busulfan treatment that the cells in the process of differentiation are cleared from the lumen of the seminiferous tubules (Nagano *et al.*, 1999). Accordingly, transplantation has generally been undertaken between week 4 and week 6 after treatment, which corresponds with the first time point in the present study. In addition, it has been reported that it requires approximately 4 weeks after transplantation for the donor cells to attach to the basal lamina and to proliferate (Parreira *et al.*, 1998; Nagano *et al.*, 1999), which corresponds to weeks 8–10 after busulfan treatment and the second time point for examination of testes in the present study. Thus, weeks 5–10 after busulfan treatment encompass the period during which transplanted spermatogonia are presumably competing with endogenous cells for space along the basal lamina.

If a higher dose of busulfan (> 20 mg kg^{-1}) depletes the testes of stem cells more effectively than 20 mg busulfan kg^{-1} , it would be expected that the rate of transplantation of ROSA26 cells in the RAG2 recipient

mice in the present study would be compromised. However, this was not the case: there was donor-derived spermatogenesis in 64% of recipients (7 of 11) examined between week 4 and week 12 after transfer with, on average, 12 tubules per section showing exogenous spermatogenesis in the positive recipients. There was also proliferation and differentiation of the transplanted cells over time. Transplantation of spermatogonia by Brinster and Zimmermann (1994) resulted in 37% colonization by genetically marked donor cells. More recent studies have reported up to 73% colonization (Avarbock *et al.*, 1996; Brinster and Nagano, 1998). The number of cross-sections typically colonized by exogenous germ cells at week 12 has been reported as approximately 12 per section (Brinster and Avarbock, 1994; Clouthier *et al.*, 1996). Other studies have found that spermatogonial proliferation and the first appearance of spermatocytes occur about one month after transplantation, with spermatids and spermatozoa appearing two or three months after transfer (Nagano *et al.*, 1999; Parreira *et al.*, 1999). Our results are consistent with these, as exogenous spermatozoa were not observed until week 8 after transfer. Thus, preparation of RAG2 mice with 20 mg busulfan kg^{-1} results in successful rates of transplantation similar to those reported in the literature with higher doses of busulfan.

A surprising outcome of the present study was the steepness of the dose–response curves with busulfan. The probability of a tubule being depleted of germ cells was $< 10\%$ at 10 mg busulfan kg^{-1} and $> 80\%$ at 20 mg busulfan kg^{-1} . Testicular masses went from maximum to minimum values between 0 mg busulfan kg^{-1} and 20 mg busulfan kg^{-1} , whereas mortality increased from 0 to approximately 50% between 20 mg busulfan kg^{-1} and 40 mg busulfan kg^{-1} . At doses for which mortality approached 50%, the variability of the testicular responses to busulfan also increased. Together, these data predict that there is a narrow range of optimal dose (20–30 mg busulfan kg^{-1}), and that slight changes in the drug, technique or animal physiology may have large effects on response. The steep dose–response curves indicate that other means should be sought to deplete recipients of germ cells in preparation for spermatogonial transplantation.

Administration of busulfan to pregnant mice may be a useful alternative to the treatment of adult males if combined with the transfer of spermatogonia to pups at day 5 after birth. Busulfan was administered to pregnant mice at day 10.5 or 12.5 after mating, at which time germ cells are proliferating in embryonic testes, and at day 14.5 or 16.5 after mating, at which time proliferation of male germ cells has ceased in mice. The dose (15 mg busulfan kg^{-1}) was intermediate between those used on pregnant rats (Hemsworth and Jackson, 1963; Gaulier and Roux, 1970) and had no noticeable effect on the health of the female mice or on the course of the pregnancy. Despite the variability in the data, spermatogenesis was

reduced at week 5 after birth in the male offspring that had been treated with busulfan *in utero* at day 10.5 or 12.5 after mating. There were no differences between treatment and control groups by week 9 after mating. One interpretation of these data is that the mice treated with busulfan *in utero* at day 10.5 or 12.5 after mating were born with fewer germ cells, and that the reduced spermatogenesis at week 5 occurs because the fewer germ cells need time to proliferate and fill the available stem cell niches before differentiation is initiated. These data also indicate that spermatogonia transplanted into 5-week-old recipients that had been treated *in utero* with 15 mg busulfan kg⁻¹ might face stiff competition by the endogenous germ cells for the stem cell niches. In the present study, the times after birth at which testes were collected after exposure to busulfan *in utero* were selected assuming that mice would need to be 5 weeks old to tolerate transplantation surgery. However, Shinohara *et al.* (2001, 2002) transplanted spermatogonia into the testes of mice on day 5 after birth and increased colonization frequency by donor spermatogonia nearly tenfold over transplantation into adult testes. In day 5 pups there are fewer germ cells and these are type A (Bellvé, 1993; Shinohara *et al.*, 2002), and junctions between Sertoli cells are also lacking (de Rooij and van Dissel-Emiliani, 1997). If *in utero* administration of busulfan to recipients does reduce the numbers of endogenous germ cells at birth, it might further increase rates of colonization by cells transplanted into pups at day 5 after birth. Experiments that examine the effects on numbers of germ cells before week 5 after birth and after administration of higher doses of busulfan to pregnant mice between day 10.5 and day 12.5 after mating are required to test this hypothesis.

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