Hormonal Protection from Procarbazine-induced Testicular Damage Is Selective for Survival and Recovery of Stem Spermatogonia

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ABSTRACT

Procarbazine produces long-term sterility in the male by killing stem spermatogonia. The degree and selectivity of protection of stem spermatogonia in rats from procarbazine by pretreatment with steroid hormones were investigated. Male LBNF rats were treated for 6 weeks with Silastic implants containing testosterone plus 17β-estradiol. The hormone-treated rats and sham-treated controls were given a single injection of graded doses of procarbazine and the hormone implants were removed the next day. Spermatogonial stem cell survival and function, assessed by the repopulation indices and sperm head counts 10 weeks later, showed that stem spermatogonia were protected by testosterone plus 17β-estradiol treatment from the toxic effects of procarbazine with a dose-modifying protection factor of about 2.5. In contrast, there was no hormonal protection from the procarbazine-induced killing of differentiating spermatogonia, preleptotene spermatocytes, and spermatocytes in meiotic prophase or from the delay in maturation of round spermatids, assessed 9 days after procarbazine injection by histological or flow cytometric methods. In addition, there was no hormonal protection from the procarbazine-induced decline in body weights and lymphocyte counts, indicating that the gastrointestinal, neurological, and hematological systems were not protected. The specificity of protection indicates that the hormonal protection of the stem spermatogonia is not the result of a systemic or overall testicular decrease in drug delivery, decrease in bioactivation, nor increase in drug detoxification, except possibly within the stem cells themselves. We conclude that the degree of hormonal protection and its specificity would be appropriate for clinical application provided that the mechanism of protection is elucidated and appears applicable to humans.

INTRODUCTION

Treatment of rats with analogues of GnRH or with gonadal steroids results in a cascade of events including decreased luteinizing hormone secretion, Leydig cell atrophy, decreased intratesticular testosterone levels, loss of Sertoli cell androgen-dependent functions, decreased estrogen secretion, Leydig cell atrophy, decreased intratesticular testosterone levels, and, to maintain the spermatocytes, the stem cells must occur surviving stem these results demonstrate that hormonal pretreatment protects stem spermatogonia from the damaging effects of procarbazine.

Chemotherapy induces sterility in male rodents and humans largely by killing stem spermatogonia (9, 10). Thus, hormonal treatment that protects these cells may minimize long-term sterility in patients undergoing cytotoxic treatments for cancer. However, extending results obtained in rats to the treatment of men is problematic because the mechanism of this protective effect is unknown. The originally proposed mechanism (11), that the hormonal treatment places the stem spermatogonia in a resting state, cannot be valid because such treatment does not alter the kinetics of spermatocyte proliferation or differentiation (12) and, to maintain the spermatocytes, the stem cells must also be proliferating.

Various alternative mechanisms may be proposed to explain the protective effect. Some of these and their consequences are listed in Table 1. Several imply that protection of multiple organs, tissues, or cell types within the testis should occur. To test whether any of these proposed mechanisms are involved, we examined whether there was protection of other tissues or other germ cells in the testis under conditions that resulted in protection of stem spermatogonia. Animal mortality, weight loss, and reduced white blood cell count were used as measures of toxicity to other tissues. Survival of differentiating spermatogonia and preleptotene spermatocytes was of particular interest as measures of toxicity to other testicular cells, because they are located adjacent to the stem cells in the basal compartment of the seminiferous tubules; if protection is mediated by the levels of active drug reaching this compartment, they should be protected as well.

MATERIALS AND METHODS

Animals. Adult LBNF rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were maintained on a 12-h/12-h light/dark cycle.

Hormone Treatment. At 15 weeks of age, the hormone-treated rats were given s.c. implants of 2-cm-long Silastic capsules containing testosterone and 0.5-cm-long capsules containing estrogen (17β-estradiol). Capsules were prepared from silicone tubing (602–305; Dow-Corning, Midland, MI) as described previously (8, 19). The hormonal treatment was continued for 6 weeks until procarbazine administration. Previous studies using Sprague-Dawley rats (1) have shown that nearly identical treatment with implants results in no change in average serum testosterone levels and a 3-fold increase in serum estradiol (45 to 125 pg/ml).

Control rats, at 12 weeks of age, were implanted with 3-cm-long Silastic capsules containing cholesterol for 2 weeks prior to injection of procarbazine. The different ages and times of implantation were used for the 2 groups so that they would be approximately weight-matched at the time of procarbazine injection. In both groups, the capsules were removed 1 day after procarbazine injection.

Procarbazine Administration. Procarbazine ([N-(1-methyllethyl)-4-(2-methylhydrazine)methyl]benzamid monohydrchloride) was a gift from Hoffmann-La Roche (Nutley, NJ). It was dissolved in normal saline and injected i.p. into rats in volumes of between 0.25 and 5 ml. To minimize possible variations due to injection technique, equal parts of the desired volume were injected at 3 sites in the peritoneal cavity. All treatment groups consisted of 8 to 10 rats.

Assessment of Reduction in Lymphocyte Counts. One day after the injection of procarbazine (at the time of removal of hormonal implants), about 500 μl of blood were withdrawn by retro-orbital sinus puncture and placed into heparinized tubes. Twenty-five μl of blood were diluted into 475 μl of 2.86% acetic acid for total white blood cell counts. Smears were prepared and Wright’s-stained for differential leukocyte counts.

Preliminary experiments showed a reduction of total white blood cell concentration between days 1 and 14 after procarbazine injection and also a drop

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5 The abbreviations used are: GnRH, gonadotropin-releasing hormone; P4, testosterone plus 17β-estradiol; RI, repopulation index; PF, protection factor; LI, diploid cell labeling index; BrdUrd, bromodeoxyuridine.

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in lymphocyte percentage between days 1 and 4. The concentrations of lymphocytes were most markedly reduced at days 1, 2, and 4 and were the most sensitive measure of procarbazine hematological toxicity. Day 1 was chosen for assay because the rats were anesthetized for implant removal at that time.

Assessment of Weight Loss. Rats were weighed 1 and 2 days before and at the time of procarbazine injection, and the average of the 3 weights was taken as the weight prior to procarbazine injection. Preliminary experiments showed that the maximum weight loss occurred at days 3 and 4 after procarbazine injection. Hence, the average weight on these 2 days minus the weight prior to injection was used as a measure of weight loss.

Histological Assessment of Spermatid Development and Survival of Differentiating Spermatogonia. Rats were killed 9 days after the injection of procarbazine, and the left testes were fixed in Bouin’s solution. Tissues were mounted in plastic (JB4 embedding kit; Polysciences, Inc., Warrington, PA), and 4-μm sections were cut and stained by the periodic acid-Schiff-hematoxylin method. From each rat, one transverse cross-section through the testis was mounted in plastic (JB4 embedding kit; Polysciences, Inc., Warrington, PA), and 4-μm sections were cut and stained by the periodic acid-Schiff-hematoxylin method. From each rat, one transverse cross-section through the testis was scanned along parallel lines 1 mm apart under a ×100 objective; the stage of the seminiferous epithelium cycle was determined for each of the first 200 tubules that came into view. In T+E-treated rats, the exact stage could not be determined reliably in stages IX to XIV tubules because development of spermatids past step 8 was markedly reduced by hormone treatment. Hence, tubules in stages IX to XIV, identified in the T+E-treated rats by 2 generations of spermatids along with pachytene spermatocytes in stage V. In each testicular section, all tubules in these stages, up to a maximum of 10, were analyzed. The nuclei of Sertoli cells showing the nucleolus were also counted, and the numbers of spermatocytes present at the indicated stages divided by the numbers of Sertoli cells were taken as measures of germ cell survival. No corrections were made to account for section thickness and differences in nuclear diameters (22), since relative, not absolute, numbers of spermatocytes per Sertoli cell were most important. Working backwards using the kinetics of spermatogenesis (23), we determined that the preleptotene spermatocytes stages VIII and the pachytene spermatocytes in stage V were A1 spermatogonia and preleptotene spermatocytes, respectively, at the time of procarbazine treatment. Therefore, the above assay should be a measure of the survival of these latter cell types (24).

For the identification of spermatogonia at a particular stage to be valid, it must be ascertained that the stages of the seminiferous epithelium cycle are unaltered by the treatments (hormonal, procarbazine). To do so, we compared the frequencies of the stages of the cycle of the seminiferous epithelium in different treatment groups of rats (analysis of variance, t test).

Flow-Cytometric Assessment of Survival of Differentiating Spermatogonia and Spermatocytes. Nine days after procarbazine treatment, rats were given injections of 30 mg/kg BrdUrd and killed 1 h later. Pieces of the right testis weighing approximately 100 mg were fixed in cold ethanol as described previously (25). The left testis was fixed in Bouin’s fluid for histological analysis.

Tests cell nuclei were prepared for flow-cytometric analysis by treatment with pepsin, acid denaturation, incubation with the monoclonal antibody IU-4 (which recognizes BrdUrd in DNA), incubation with a fluorescein isothiocyanate-conjugated antibody, and staining for DNA with propidium iodide (26). A total of 50,000 cells were analyzed in each sample for both DNA content and BrdUrd incorporation (25). Histograms were analyzed using the “Multi” series software (Phoenix Flow Systems, San Diego, CA). The average coefficient of variation of the diploid cell peak was 2.4%.

Since the diploid peak contains primarily nonproliferating, nongermline cells, which are not lost as a result of hormone treatment and are not killed by procarbazine, it was used for normalization. The small number of G1 spermatogonia and preleptotene spermatocytes and secondary spermatocytes contained within the diploid peak should have only a small quantitative effect on the results.

One-dimensional DNA histograms were fitted using the MultiCycle program. The numbers of cells in the 1C peak, 2C peak, S-phase region between 2C and 4C, and 4C peak were calculated. The 4C peak consists mainly of primary spermatocytes in meiotic prophase, plus a small number of G2 spermatogonia. The ratio of 4C to 2C cells was used as a measure of the numbers of primary spermatocytes in meiotic prophase (i.e., those between the preleptotene stage and meiotic divisions).

BrdUrd-labeled cells counted by flow cytometry can be used as a measure of spermatogonial survival (27). The labeled cells should be mostly preleptotene spermatocytes (50%), B spermatogonia (25%), and intermediate spermatogonia (12.5%), with various types of A spermatogonia comprising the remaining 12.5% of the labeled cells.

Bivariate histograms were analyzed using the Multi2D program to calculate the numbers of cells in a given area of the histogram. A diploid cell LI is a percentage calculated by dividing the number of cells that were labeled (all diploid cells) by the total number of diploid cells (labeled and unlabeled). Since there were some aggregates (clumps) of diploid cells with haploid cells that were not excluded by the analysis gate, 3C signals (a diploid cell plus a haploid cell) were included with the unlabeled diploid cells and signals between 4C and 5C (5-fluorodeoxyuridine cells plus a haploid cell) were included with the labeled cells.

Assessment of Stem Spermatogonial Survival and Recovery. Rats were killed 10 wk after procarbazine injection. The left testis was fixed in Bouin’s fluid and processed for histology as described previously (28). The histological sections were stained with iron-hematoxylin, and the RL, which is the percentage of tubules showing repopulation, was determined. Tubules were considered to be repopulating if they contained at least 3 cells at the B spermatogonia stage or later. The tunica was removed from the right testis, the testis was weighed, and the tissue was processed for counts of sperm heads (nuclei of sonication-resistant elongated spermatids) (28). Sperm heads were counted using a hemacytometer.

Protection Factors. The degree of protection was quantified by PF (8). The PF is the ratio of the procarbazine dose required to produce a given effect

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**Table 1** Some of the possible mechanisms to explain the protection of stem spermatogonial survival/function from procarbazine-induced damage with hormonal treatment

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Reason for considering</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alteration of systemic metabolism of procarbazine</td>
<td>Liver function is affected by androgens (13)</td>
<td>Protection of multiple organs and tissues</td>
</tr>
<tr>
<td>Decreased drug delivery caused by a decrease in testicular blood flow</td>
<td>LH regulates testicular blood flow (14)</td>
<td>Protection of all cells within testis</td>
</tr>
<tr>
<td>Decreased bioactivation of procarbazine within testis</td>
<td>Procarbazine requires bioactivation involving P-450 enzymes and molecular oxygen (15)</td>
<td>Protection of multiple cell types within testis</td>
</tr>
<tr>
<td>Reduced levels of thiols within testis</td>
<td>Sulphydryl reducing agents detoxify active metabolites of procarbazine (17)</td>
<td>Protection of multiple cell types within testis</td>
</tr>
<tr>
<td>Increase in number of stem spermatogonia at time of procarbazine treatment</td>
<td>Stem cells may increase in number in response to depletion of differentiated cells (18)</td>
<td>Protection would be specific for stem cells; protection factor should decrease at higher doses</td>
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</table>
when the protective treatment is given to the dose necessary to produce the same effect in control (cholesterol-treated) rats. The PF was computed at data points obtained in the T+E-pretreated rats. The dose to produce the same effect in control rats was determined graphically by the intersection of a horizontal line through the data point in the hormone-treated rats with the straight line drawn connecting points on the dose-response curves for the control rats.

RESULTS

The weights of the cholesterol- and T+E-treated rats at the time of procarbazine injection were very nearly matched. The cholesterol-treated rats weighed $333 \pm 20$ (SD) g, while the T+E-treated rats weighed $346 \pm 18$ g.

No cholesterol-pretreated rats died as a result of procarbazine injection at doses up to the maximum of 300 mg/kg. No T+E-pretreated rats died at procarbazine doses up to 340 mg/kg, but 4 of 6 rats receiving 380 mg/kg died. All deaths occurred within 48 h after injection. Since all control animals survived 300 mg/kg and more than half of the T+E-pretreated animals died at 380 mg/kg, there cannot be much protection against procarbazine-induced lethality with T+E treatment (\(PF < 1.3\)).

Survival and Recovery of Stem Spermatogonia. The survival and recovery of stem spermatogonia were assessed by testis weight measurements, counts of repopulating tubule cross-sections, and sperm head counts at 10 weeks after administration of graded doses of procarbazine. The results are shown in Fig. 1.

Protection was observed with all 3 endpoints. At 160, 220, and 300 mg/kg, all values obtained in T+E rats were higher than those obtained for the cholesterol-treated rats.

PFs were between 2.4 and 2.7 for testis weight, at least 2.7 for repopulation index, and 2.3 to 2.6 for sperm head counts. The value of the PF for repopulation index is likely to be appreciably higher than 2.7 (300/110) since at the highest dose for which we have adequate data in the T+E rats (300 mg/kg), the RI was $98 \pm 2\%$ (SEM), while at the lowest dose used for the control rats (110 mg/kg) the RI was $82 \pm 6\%$. Furthermore, the 6 T+E-pretreated rats that survived following procarbazine doses of 340 or 380 mg/kg had RIs of $\approx 99\%$.

Body Weight Changes. To examine whether T+E treatment induced systemic protection, body weight losses at 3 to 4 days following procarbazine injection were measured. Control rats showed a dose-responsive change in the percentage of initial body weight lost, reaching a decline of about 14% at doses of 300 mg/kg. The decline in body weight in T+E-pretreated rats was almost identical. PFs ranged from 1.1 to 1.4 in the dose range of 100 to 300 mg/kg (Fig. 2a). Since the T+E-pretreated rats had a slightly greater average initial body weight at the time of procarbazine injection (346 g versus 333 g for the control rats), the weight loss data were also expressed as the absolute loss of body weight in grams (data not shown). In this case, the PFs were between 1.0 and 1.1 over most of the dose-response curve, except for the highest dose point. Thus, we conclude that T+E pretreatment offers no significant protection against procarbazine-induced loss in body weight.

Hematological Changes. The systemic effects of procarbazine and the effect of hormonal pretreatment were also assessed by performing measurements of total white blood cell counts and the percentage of lymphocytes in control and T+E-treated rats. T+E treatment reduced the total white blood cell count from $12,200 \pm 700$ (SEM)/mm$^3$ to $9,700 \pm 500$/mm$^3$ and the percentage of lymphocytes from $92.3 \pm 0.8\%$ to $88.1 \pm 1.0\%$ (and hence a decline in the lymphocyte count from $11,200 \pm 700$/mm$^3$ to $8,500 \pm 400$/mm$^3$). The scales on which the dose-response curves (Fig. 2b) are plotted are therefore adjusted so that the values at zero dose overlap.

In control animals, procarbazine resulted in a dose-responsive decline in lymphocyte counts at 1 day after injection to about 18% of normal at 300 mg/kg (Fig. 2b). An essentially identical decline to 23% was observed with this dose of procarbazine in T+E-pretreated rats. PFs along the dose-response curve from 100 to 300 mg/kg ranged from 0.7 to 1.2, indicating that hormonal pretreatment did not protect the rats against the hematological toxicity of procarbazine.

Development of Spermatids. Since no systemic protection from procarbazine toxicity was found, we next investigated whether other testicular cells besides the stem cells were protected. To assess this by histological means, it was first necessary to ascertain that the frequencies of stages of the cycle of the seminiferous epithelium were not altered by the hormone treatment. The relative numbers of tubules found in each of stages I-VIII and in stages IX-XIV combined in cholesterol-treated and in T+E-pretreated rats were not significantly different (\(t\) test).

The frequencies of all these stages were not affected by procarbazine at doses up to 65 mg/kg in the cholesterol-treated rats and up to 40 mg/kg in T+E-pretreated rats at 9 days after drug treatment. In the

Fig. 1. Dose-response curves for stem spermatogonial survival and recovery assayed by (a) testis weights, (b) seminiferous tubule repopulation indices, and (c) sperm head counts after procarbazine administration to cholesterol- and T+E-pretreated rats. Bars, SE. PF were calculated at several isoeffect levels.
by procarbazine at 65 mg/kg; however, stages V through VIII were all T+E-pretreated rats. The lymphocyte counts for the cholesterol- and T+E-pretreated rats increased. There was a concomitant decrease in tubules in stages IX to XIV in the T+E group. These results indicate that this dose of procarbazine delays the development of spermatids that would normally have progressed beyond step 8 during the 9-day interval, resulting in an accumulation of tubules with spermatids at steps 5 to 8. Since this effect of procarbazine was noted at 65 mg/kg in T+E-treated rats but not in cholesterol-treated rats, we conclude that hormonal treatment actually increases the sensitivity of spermatids to induction of developmental delays.

**Survival of Differentiating Spermatogonia (Histology).** Counts of preleptotene and pachytenes spermatocytes were performed 9 days after procarbamine injection to determine whether there was protection of differentiating spermatogonia by the hormone treatment. However, it was first necessary to determine whether the hormone treatment had any effect on the numbers of these cells in the absence of procarbazine treatment. The number of preleptotene spermatocytes per Sertoli cell nucleus was 4.31 ± 0.09 in control rats and similarly was 4.12 ± 0.23 in the T+E-treated group. The numbers of pachytenes per Sertoli cell was decreased from 5.22 ± 0.08 in the control group to 3.38 ± 0.21 in the T+E group. Because of the hormone effect on the numbers of spermatocytes before procarbazine treatment, the ordinates of the dose-response curves were adjusted (Fig. 3) so that the values at zero dose overlapped.

Dose-response curves for the effect of procarbazine on spermatocyte counts (Fig. 3) showed that 40 mg/kg reduces the numbers of preleptotene and pachytenes spermatocytes similarly in both cholesterol- and T+E-pretreated rats. At procarbazine doses of 65 mg/kg, the rats pretreated with T+E had significantly lower counts of spermatocytes than those receiving cholesterol. Since the preleptotene and pachytenes spermatocytes scored had been A2 spermatogonia and preleptotenes at the time of treatment, we conclude that these latter cells are sensitive to procarbazine and exhibit no evidence of hormonal protection. In fact, hormone treatment may even sensitize the differentiating spermatogonia and preleptotene spermatocytes to procarbazine.

**Survival of Differentiating Spermatogonia and Primary Spermatocytes (Flow Cytometry).** Because of uncertainties in the histological assay of spermatogonia or spermatocytes at procarbazine doses of 65 mg/kg and above due to its effects on staging, flow-cytometric assays were also performed. The flow-cytometric DNA histogram from cholesterol-treated (control) rats (Fig. 4a) shows 3 major peaks, at 1C, 2C, and 4C DNA contents, corresponding mainly to early (noncondensed) spermatids, somatic cells, and spermatocytes in meiotic prophase, respectively. The region between the 2C and 4C peaks contains cells with S-phase DNA content (preleptotene spermatocytes...
and spermatogonia). The bivariate histogram shows peaks of unlabeled cells at 1C, 2C, and 4C and BrdUrd-labeled cells uniformly distributed between 2C and 4C (Fig. 4b).

The most apparent effect of procarbazine on the DNA histogram of testis cells from control rats was to decrease the number of 4C cells (Fig. 4c). However, there was a nearly concomitant increase in cells with S-phase (particularly late S) DNA content. The bivariate histogram (Fig. 4d) shows that the increase in cells with late S-phase DNA content was due primarily to unlabeled cells with late S-phase DNA content. Note also that most of the remaining labeled cells also had late-S DNA content. We interpret the increase in late S as a procarbazine-induced delay in the completion of S-phase of the preleptotene.

Fig. 4. Flow-cytometric histograms of testis cell nuclei prepared from rats 9 days after receiving injections of procarbazine. a, c, e, and g, DNA histograms, with cells with haploid (1C), diploid (2C), S-phase (S), and tetraploid (4C) DNA content indicated. b, d, f, and h, bivariate BrdUrd-DNA histograms represented as contour plots with BrdUrd-labeled S-phase cells (SL), unlabeled late S-phase (late SU), and minor peaks of clumped cells (*). a and b, control rats (cholesterol-pre-treated, 0 procarbazine); c and d, 250 mg procarbazine/kg body weight (cholesterol-pre-treated, 0 procarbazine); e and f, T+E-pre-treated rats (0 procarbazine); g and h, T+E-pre-treated rats given 250 mg procarbazine/kg body weight. Bars, SE.
spermatocytes; some of these cells were still synthesizing DNA while most had ceased DNA synthesis.

Despite this increase in cells with S-phase DNA content, the bivariate histograms from procarbazine-treated rats (Fig. 4d) show a decrease in the LI. In particular, there was a loss of cells with early and mid-S-phase DNA contents. We interpret this result as indicating a loss of cells from all parts of S-phase but with the addition of another population whose exit from S-phase was delayed by the procarbazine treatment.

The DNA histograms from T+E-treated rats (Fig. 4e) indicate that, compared with control rats, there was a slight reduction in cells in the 4C peak, and a significant reduction in the 1C peak (from 118 ± 9% of the 2C peak in control to 26 ± 4% in T+E treated). This is consistent with the known slight reduction in numbers of pachytene spermatocytes and marked reduction in spermatids as a result of hormone treatment (29). Analysis of the bivariate histogram (Fig. 4f) shows that there was no reduction in the LI as a result of hormone treatment (Fig. 5b). This confirms that the hormone treatment did not put spermatogonia into a resting state.

The most dramatic effect of procarbazine on the T+E-treated rats shown in the DNA histogram (Fig. 4g) was the reduction in the 4C peak (plotted in Fig. 5a), indicating loss of most primary spermatocytes. The most dramatic effect of procarbazine seen from the bivariate histogram (Fig. 4h) was a reduction in the LI (plotted in Fig. 5b). The procarbazine-induced losses of cells with 4C DNA content and those that were synthesizing DNA were greater in the T+E-treated rats than in controls.

**DISCUSSION**

The purpose of this study was 3-fold. (a) We wanted to quantify the hormonal protection of spermatogonial stem cell survival/recovery from the cytotoxic effects of procarbazine. (b) We wished to determine whether the protection was specific for the testis in general and the stem spermatogonia in particular. (c) We wanted to utilize these data to test some of the possible mechanisms of stem spermatogonial protection.

Using 2 endpoints of stem spermatogonial survival, RI and sperm head counts, protection factors in the range of 2.5 to 3 were obtained. These values are consistent with the rough estimates made earlier (8) based on partial dose-response curves. PFs of 2.5 to 3 are encouraging. If similar protection were obtained in a clinical situation, hormonally pretreated patients could be treated with procarbazine at 6000 mg/m² and still display no more long-term testicular toxicity than those treated with only 2000 to 2400 mg/m² without hormones. Since about 90% of patients treated with 6 cycles of mechlorethamine-vincristine (Oncovin)-procarbazine-prednisone chemotherapy (procarbazine dose, 6000 mg/m²) were azoospermic at a median of 3 years after treatment but none treated with about 2000 mg/m² was azoospermic at 2.5 years (30), this degree of protection would be beneficial to patients. Whether or not this degree of protection is achieved should be testable in a clinical trial.

Because no other authors have generated dose-response curves to allow calculation of PFs, we cannot compare these results. However, we can compare the amounts of protection in the studies of Ward et al. (5) and Glode et al. (6) at doses that yielded similar RIs to points along our dose-response curve. Averaging the results of Ward et al. (5) obtained at 50 and 90 days (we used 70 days), we calculate a RI of about 5% in the procarbazine-treated piebald variegated rats and 94% in the same rats that had been pretreated with GnRH prior to procarbazine treatment. At a procarbazine dose of 300 mg/kg, we obtained a RI of 4% in our strain of rats; pretreatment with T+E raised the RI to 98%. Thus, the amount of protection obtained with GnRH agonist pretreatment was comparable to that obtained here using T+E pretreatment. In contrast, the degree of protection reported by Glode et al. (7), who also used T+E, was less than that found here. They found a RI of 2% in the control Sprague-Dawley rats receiving 10 injections of procarbazine but only 25% in the T+E-treated rats. The lower protection may be a result of the use of outbred versus inbred rats (5) or the variability observed in the response of Sprague-Dawley rats to procarbazine (8), which would affect the near-zero values in the control more than those in the protected rats.

The second question, whether protection from the systemic toxicities of procarbazine would be observed with the hormonal treatment, is important because if systemic protection were observed, changes in hepatic metabolism of procarbazine might be involved and protection of tumors would be likely. Procarbazine is known to be toxic to the neurological, gastroenterological, and hematopoietic systems (31). The death of some rats within 48 h of injection of high doses of procarbazine appears to be a result of the neurotoxicity. The weight loss is likely a result of both the gastrointestinal toxicity and neurotoxicity of procarbazine. The rats were noticeably lethargic for about 1 day after procarbazine injection, and this likely affected their food intake; subsequently many developed diarrhea. Although hematological toxicity has been generally ascribed to killing of hematopoietic cells in the bone marrow (31), we found that the most sensitive endpoint for hematological toxicity was the decline in lymphocyte count at 1 or 2 days after treatment. This is most likely due to chemotherapy-induced apoptotic death of mature lymphocytes (32) and not to death of lymphocyte precursors. There was no protection by hormonal pretreatment against either the procarbazine-induced lethality, body weight loss, or the drop in lymphocyte count.
Several endpoints of testicular damage by procarbazine were examined. Histological assays showed that hormonal pretreatment had no significant effects on the sensitivities of A1 spermatogonia or preleptotene spermatocytes at doses of 40 mg/kg, which is the highest dose at which precise staging was possible in T+E-treated rats. At 65 mg/kg, the cells were actually more sensitive in the T+E-pretreated animals; however, there could have been some error in staging because of the delay in spermatid maturation in the T+E-pretreated rats at this procarbazine dose. Any such delay in spermatid maturation would result in the spermatogonia and spermatocytes actually being at a later stage than they appeared based on their association with spermatids. But this shift in staging cannot account for the increased procarbazine sensitivity of these cells in the T+E-pretreated rats, because if the cells were at stages just later than A1 or preleptotene they should have become more resistant.

Flow cytometry allowed independent and quantitative analysis of cells based on DNA content or DNA synthetic abilities without relying on staging tubules. We anticipated that the numbers of 4C cells would provide a measure of meiotic primary spermatocytes and that the LI would provide a measure of preleptotene spermatocytes and spermatogonia. The apparent delay in completion of S-phase of the preleptotene spermatocytes made this distinction less clear.

The loss of 4C cells in the DNA histograms was a result not only of the killing of 4C primary spermatocytes and of type A2 spermatogonia through preleptotene spermatocytes that would have progressed to meiotic prophase within 9 days, but also of the blockage of surviving preleptotene spermatocytes in premeiotic DNA synthesis. In either case, the loss of 4C cells measures the toxicity of procarbazine on differentiating spermatogonia and early primary spermatocytes.

The LI obtained from the bivariate histograms measures not only the surviving intermediate and B spermatogonia and preleptotene spermatocytes, but also the spermatocytes in meiotic prophase that were still in DNA synthesis. The cells being measured in the LI assay were therefore A1 through intermediate spermatogonia at the time of procarbazine treatment. However, the reduction in LI is an underestimate of the killing of differentiating spermatogonia because of the contribution of meiotic spermatocytes still completing S-phase.

Analysis of both the 4C cells from the DNA histograms and LI from the bivariate histograms shows that the differentiating spermatogonia and early primary spermatocytes are more sensitive in the hormone-pretreated rats than in nontreated rats (Fig. 5). The uncertainties as to the stage of cells with S-phase DNA content after procarbazine treatment do not affect this conclusion, as we performed the measurements objectively in both cases. It should be emphasized that stem spermatogonia constitute only 1.3% of the spermatogonia plus preleptotene spermatocytes, and their labeling index is only 10%, which is lower than that of the later stages of spermatogonia or preleptotene spermatocytes. Thus, the stem spermatogonia contributed negligibly to the labeled cells assayed by flow cytometry; a similar argument can be used to show that stem cells contributed negligibly to the 4C peak.

The greater sensitivity of spermatids to procarbazine-induced toxicity in the hormone-treated animals may be a result of the androgen deprivation, which alone results in many of them degenerating at step 7 anyway (33). The greater sensitivity of the spermatogonia and preleptotene spermatocytes was unexpected, since these cells are hardly affected by the hormonal treatment. In either case, there was no evidence of hormonal protection of any of these cells from the cytotoxic or delaying effects of procarbazine.

In contrast, differentiating spermatogonia and spermatocytes have been shown to be protected from the mutagenic effects of procarbazine; there were fewer postimplantation deaths of fetuses in female rats mated to males treated with medroxyprogesterone and testosterone prior to procarbazine (6). The use of a different strain of rat or the use of medroxyprogesterone instead of estradiol along with testosterone in the suppression protocol could be responsible for this difference, but these explanations seem unlikely. The following conclusions are based on our observations of selective protection of stem spermatogonia; the different results of Veléz de la Calle and Jegou (6) remain as a caveat in the arguments.

The results obtained here address the possible mechanisms of hormonal protection of stem spermatogonia from procarbazine-induced damage. One mechanism, an alteration in the systemic metabolism of procarbazine, is unlikely since there was no protection against lethality or loss of weight or lymphocyte count. Although it is not certain that the same metabolite of procarbazine is responsible for both the stem spermatogonial toxicity and the other toxicities, metabolism of procarbazine to the azyoxyprocarbazine isomers is required for all proposed pathways of toxicity (17, 34). Therefore, systemic metabolism to this level cannot be affected by the hormonal treatment. A decrease in testicular blood flow, resulting in delivery of less procarbazine to the testis, is also unlikely to be the mechanism by which stem spermatogonia are protected, because other germinal cells, the differentiating spermatogonia, primary spermatocytes, and round spermatids, are not protected.

Other possible mechanisms of protection include decreased bioactivation or increased detoxification of procarbazine within cells of the testis. If these occurred in nongerminall cells such as the Leydig, peritubular, or Sertoli cells through which the drug might pass and be metabolized before reaching the germinal cells, then all of the germinall cells, and particularly the stem and differentiating spermatogonia and preleptotene spermatocytes, which are adjacent to each other in the basal compartment of the seminiferous tubules, should be protected equally. That they were not indicates that the mechanism of protection cannot involve changes in procarbazine metabolism elsewhere in the testis; however, these experiments do not rule out the possibility that the hormonal treatment selectively alters the ability of the stem cell to bioactivate procarbazine or to detoxify it, perhaps through an intracellular thiol, such as glutathione. In any case, mechanisms involving drug delivery or bioactivation of procarbazine are unlikely to be the dominant cause of protection since hormonal treatment protects stem spermatogonia from radiation damage as well.

Another possible mechanism is that under conditions of hormonal suppression of the completion of spermatogenesis, the number of stem cells increases, perhaps as a homeostatic response to the loss of differentiated cells. If the number of cells increases without any change in their individual procarbazine sensitivities, the dose-response curve for stem cell survival would be shifted upwards and to the right by the hormonal treatment without a change in slope. A consequence of such a shift would be a decrease in the PF with increasing dose. Although the present data are not sufficient to unequivocally rule out a decrease in PF with increasing dose, the PFs appear to be relatively constant with dose (Fig. 1), indicating that there is a change in the sensitivity of the stem cells, and not merely an increase in their number. The observation that stem spermatogonia are protected from the mutagenic effects of procarbazine by hormonal treatment (6) also argues against the protection of stem cells being due solely to an increase in their numbers; rather it indicates that the amount of DNA damage fixed in these cells is reduced.

Therefore, the suggested mechanisms of hormonal protection from procarbazine-induced damage listed in Table 1 are considered unlikely. Other possible mechanisms of protection, besides specific bioactivation or detoxification of procarbazine in stem cells, include increased DNA repair within stem cells, decreased stem cell sensitiv-

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6 N. Parchuri and M. L. Meistrich, unpublished observations.

7 B. Kurdoglu, G. Wilson, W-S. Ye, N. Parchuri and M. L. Meistrich. Protection from radiation-induced damage to spermatogenesis by hormone treatment, submitted for publication.
ity to procarbazine induced by altered secretion of cytokines by Sertoli cells (35), subtle cell cycle alterations of stem cells, and increased rate of recovery of spermatogenesis from an unaltered number of surviving stem spermatogonia. Further experiments are required to test these possibilities.

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Hormonal Protection from Procarbazine-induced Testicular Damage Is Selective for Survival and Recovery of Stem Spermatogonia

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