Protection from Procarbazine-induced Testicular Damage by Hormonal Pretreatment Does Not Involve Arrest of Spermatogonial Proliferation

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ABSTRACT

Hormone treatments that suppress sperm production enhance the recovery of spermatogenesis after gonadal exposure to various cytotoxic agents. It has generally been assumed that the mechanism of protection involved an arrest of spermatogonial kinetics. To test this hypothesis critically, we examined spermatogonial kinetics and numbers in rats in which the completion of spermatogenesis was suppressed with a 6-week testosterone plus 17β-estradiol treatment that protected the testis from procarbazine-induced damage. Histological examination showed that the numbers of A-aligned, intermediate, and B spermatogonia and preleptotene spermatocytes and their mitoses were unaffected by testosterone plus 17β-estradiol treatment. Flow cytometric analysis of bromodeoxyuridine-labeled cells showed that the percentage of diploid cells undergoing DNA synthesis, the progression of B spermatogonia and preleptotene spermatocytes through S-phase, the division of intermediate and B spermatogonia, the entry of intermediate spermatogonia into their next S-phase as type B cells, and the progression of cells through meiotic prophase were either unchanged or very slightly increased. Thus, changes in spermatogonial numbers or suppression of their proliferation cannot account for protection of spermatogenesis from exposure to cytotoxic agents.

INTRODUCTION

Sterility is a frequent side effect of chemotherapy or radiotherapy for cancer. Procarbazine, one of the most effective chemotherapeutic drugs in the treatment of Hodgkin's lymphoma, produces in men prolonged testicular damage that is manifested by the failure of spermatogenesis to recover after drug treatment (1). Similarly, cyclophosphamide (2) and irradiation to the region around the gonads (3) produce extensive testicular damage. Pretreatment of rats with gonadal steroids (4, 7–12), gonadotropin-releasing hormone agonists (13, 14), or antagonists (15) can protect the testis against the damaging effect of procarbazine as well as that produced by irradiation (6, 16–18) and cyclophosphamide (5). These observations produced some initial optimism that similar procedures could be applied to men, but such attempts have been unsuccessful; a better understanding of the mechanisms of protection in the rat is needed to determine how and if this procedure can be properly applied.

The originally proposed mechanism by which the hormonal treatment was supposed to protect the testis was by interruption of the pituitary-gonadal axis, reducing the rate of spermatogenesis and rendering the resting testis more resistant to the effects of chemotherapy (19). Because the target cells for prolonged reduction in spermatogenesis are generally considered to be the spermatogonia (20), most studies have assumed that hormonal protection was due to rendering the spermatogonia quiescent (4, 6, 8, 9, 13, 16).

An alternative and quite opposite mechanism was proposed in studies showing that FSH (4) pretreatment protected spermatogenesis in the rhesus monkey from radiation-induced damage (21). FSH treatment increased the numbers of A-type spermatogonia, the proliferating stem cells, before irradiation (22). Based on studies in the mouse showing that proliferating spermatogonial stem cells were more resistant than quiescent stem cells to irradiation (23), it was suggested that FSH-stimulated proliferation of the stem cells rendered them more resistant. It is also possible that FSH treatment simply produced more stem cells that were available for recovery. This mechanism might be applied to explain protection in the rat in which sperm production was hormonally suppressed; the absence of more mature cells can relieve feedback inhibition of undifferentiated spermatogonial proliferation, resulting in an increase in spermatogonial numbers (24).

We have argued that arrest of spermatogonial kinetics was unlikely to be the mechanism of protection because complete elimination of gonadotropins and nearly total ablation of androgens by hypophysectomy do not alter the kinetics of spermatocyte development (25). Nevertheless, there is still widespread belief that kinetic suppression is indeed responsible for the protection of recovery of spermatogenesis. To demonstrate conclusively that this hypothesis is not valid, we show here that spermatogonial proliferation is not suppressed by hormonal perturbations that result in protection of the recovery of spermatogenesis after cytotoxic insult. Furthermore, the numbers of undifferentiated A spermatogonia are unchanged, indicating that an increase in stem cell number at the time of administration of the cytotoxic agents is unlikely to be responsible for the enhanced recovery.

MATERIALS AND METHODS

Animals. Adult LBNF1 male rats obtained from Harlan Sprague Dawley (Indianapolis, IN) were maintained on a 12-h/12-h light/dark cycle. They were allowed to acclimate for at least 10 days before initiation of experiments, at which time they were 11–12 weeks of age.

Hormone Treatment. The hormone treatment (T+E) involved implantation of capsules containing testosterone and capsules containing estradiol-17β s.c. Capsules of the indicated lengths were prepared from Silastic silicone tubing as described previously (10, 26). Control rats were implanted with 3-cm-long Silastic capsules containing cholesterol. Rats were maintained on the hormonal treatment for 6 weeks to achieve maximal protection against procarbazine (27). Some were then killed for histological analysis of spermatogenesis, and other rats were given either BrdUrd for kinetic analysis or procarbazine injections to assess protection of spermatogenic recovery. The BrdUrd-injected rats had the hormone-containing Silastic capsules left in them until they were killed so that the kinetics of spermatogenesis could be examined under the hormonal conditions used to achieve protection. The procarbazine-treated rats had the capsules removed 1 day after procarbazine injection, as done previously in studies of protection by hormonal pretreatment (10, 11).

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4 The abbreviations used are: FSH, follicle-stimulating hormone; T+E, testosterone plus 17β estradiol; BrdUrd, bromodeoxyuridine; RM, relative movement.
Histological Counts of Spermatogonia. The left testes of four control rats and four rats treated for 6 weeks with T+E were fixed in Bouin's fluid for histological analysis. Tissue was embedded in plastic (JB4; Polysciences, Warrington, PA), and single 4-μm sections were stained with periodic acid-Schiff hematoxylin. Tubules were classified into stages of the cycle of the seminiferous epithelium (28), and A spermatogonia, mitotic figures, differentiating spermatogonia or preleptotene spermatocytes, and Sertoli cells were counted in eight tubules in each of stages II through VII in each testis. In these stages, the A spermatogonia are undifferentiated. The differentiating cells counted were intermediate spermatogonia in stages II to IV, B spermatogonia in stages IV to VI, and preleptotene spermatocytes in stages VI and VII. Sertoli cells were counted if the nucleus was seen in the section. No corrections were made to account for differences in nuclear diameters (29) because the differences between control and hormone-treated testes were most important, not the absolute values of the cell numbers.

Cell Kinetic Analysis by Flow Cytometry. For cell kinetic analysis, control and T+E-treated rats were injected i.p. with BrdUrd at 30 mg/kg. Rats (6 rats/group) were killed 0.5, 8, 16, 27, and 406 h later, and testicular cells fixed in ethanol as described previously (30).

Testis cell nuclei were prepared for flow cytometric analysis by treatment with pepsin, acid denaturation, incubation with an undiluted monoclonal antibody to BrdUrd in DNA (Clone B44, Cao. No. 347580; Becton Dickinson Immunocytometric Systems, San Jose, CA), and staining with a FITC-conjugated antibody, and staining for DNA with propidium iodide (31). Bivariate DNA/BrdUrd histograms, similar to those shown previously (11), were analyzed using the Multi series software (Phoenix Flow Systems, San Diego, CA).

The cellular composition of the peaks in the one-dimensional DNA histograms was deduced from the known DNA contents of testicular cells and had already been confirmed using separated cells (32, 33). The 2C peak contains primarily nonproliferating germinal cells, plus some G1 spermatogonia and preleptotene spermatocytes and a few secondary spermatocytes. The 4C peak consists mainly of primary spermatocytes, from the leptotene stage to the first meiotic division, plus a few G1 spermatogonia. The 1C peak consists of round and possibly elongating spermatids; condensed spermatids take up less propidium iodide and are found below the 1C peak.

The region of each bivariate histogram containing the BrdUrd-labeled cells (positive green fluorescence) was projected onto the X axis to obtain the DNA distribution of the labeled cells. The parameters of these distributions were then calculated as described below.

The RM at time t of the labeled cells that remain undivided is a measure of the progression of cells through S-phase and is defined as follows (30):

$$RM(t) = \frac{C(2C)(t) - C(2C)}{C(4C) - C(2C)}$$

where $C(2C)$ is the mean red fluorescence channel (DNA content) of the 2C (G2) cells, $C(4C)$ is the mean red fluorescence channel of the 4C (G2M) cells, and $C(i)$ is the mean red fluorescence channel of the BrdUrd-labeled cells that have not yet divided by time t.

The fraction of labeled cells that have divided ($f^{id}$) was calculated for the testicular cell system as follows:

$$f^{id}(t) = \frac{N^d_t}{N^d}$$

where $N^d$ and $N^d_t$ are the numbers of labeled undivided cells and labeled divided cells, respectively, and the latter are divided by 2 because two cells are produced from each cell division. This definition is more appropriate for this system than the one originally used for cultured or tumor cells, where the correction for cell division was applied after calculating $f^{id}$ (30, 31). When it was not possible to unequivocally resolve the labeled divided cells from the labeled undivided cells (e.g., at 42 h, when many of the divided cells had completed their second S-phase and were approaching G2 DNA content), $N^d_t$ was calculated as the number of labeled cells with DNA content below a line drawn at the S-phase–G2M boundary. However, this procedure may underestimate the true value of $N^d$.

The fraction of labeled cells that have divided and have entered their next S-phase ($f^{id}_{s}$) was calculated as follows:

$$f^{id}_{s}(t) = \frac{N^{d}_{s}}{N^d}$$

where $N^d_s$ is the number of divided cells that are in the S-phase region of the DNA histogram.

The fractions of labeled cells that have passed through the first meiotic division, $f^{s}_{mI}$, and through both meiotic divisions, $f^{s}_{mII}$, are measures of the durations of the meiotic prophase and the secondary spermatocyte stage, respectively. The numbers of labeled cells in the 1C peak ($N^d_{1C}$, round spermatids), in the 2C peak ($N^d_{2C}$, secondary spermatocytes), and in the 4C peak ($N^d_{4C}$, primary spermatocytes), of the bivariate histograms obtained at 406 h (16.9 days) after BrdUrd injection were used to calculate $f^{s}_{mI}$ and $f^{s}_{mII}$ as follows:

$$f^{s}_{mI}(t) = \frac{N^d_{2C}/2 + N^d_{1C}/4}{N^d_{4C} + N^d_{2C}/2 + N^d_{1C}/4}$$

$$f^{s}_{mII}(t) = \frac{N^d_{1C}/4}{N^d_{4C} + N^d_{2C}/2 + N^d_{1C}/4}$$

where $N^d_{2C}$ and $N^d_{1C}$ were divided by 2 and 4, respectively, because one primary spermatocyte produces two secondary spermatocytes and four round spermatids.

Cell Kinetics Models. In control animals, the labeled cells include preleptotene spermatocytes, B spermatogonia, intermediate spermatogonia, and various types of A spermatogonia. The cell cycle phase durations for the cells that comprise more than 5% of the labeled cell population are presented in Table 1.

The expected RM of labeled undivided cells ($RM^b$) was calculated for each of the spermatogonial categories and preleptotene spermatocytes using the durations of the S-phase and G2M phases (Table 1) and equations (Equation 4 in Ref. 38) for the RM of a nongrowing population of cells (i.e., one with a rectangular age distribution). Equation 4 from that paper was used after correction of an erratum; for $t \leq T_{G2M}$, $RM^b(t) = \frac{1}{2} + \frac{t}{T_{G2M}} - \frac{t^2}{2(T_{G2M})^2}$ (the final $a^2$ was omitted in the original paper), in which $T_S$ is the duration of the S-phase of the cell cycle.

The fractions of labeled cells that should pass through the first and second meiotic divisions at various times were calculated from the numbers of labeled preleptotene spermatocytes that passed through these divisions because none of the cells labeled as B spermatogonia or at earlier stages are expected to do so within 406 h. The labeled preleptotene spermatocytes pass through the first meiotic division when they reach the middle of stage XIV in the next cycle and pass through the second meiotic division when they reach the end of stage XIV, which should commence 392 h and 402 h after labeling, respectively (25). The denominator, which is the total number of labeled cells, was calculated from the sum of cells labeled as preleptotene spermatocytes, and B, intermediate, and A spermatogonia. The numbers of B and intermediate spermatogonia were multiplied by 2 and 4, respectively, because they have

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Percentage of labeled germ cells of each type</th>
<th>Cell cycle phase durations (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate</td>
<td>8.9%</td>
<td>13°</td>
</tr>
<tr>
<td>B</td>
<td>19.3%</td>
<td>13°</td>
</tr>
<tr>
<td>Preleptotene</td>
<td>59.6%</td>
<td>68.1°</td>
</tr>
</tbody>
</table>

Values are based on published figures for Sprague Dawley rats; interstrain variations are negligible (34).

b The relative number of labeled cells in these cell categories were calculated by multiplying the numbers of these cells (35) by the durations of their S-phases (36).

c Taken from the work of Huckins (36).

d Calculated from the stage of the cycle of the seminiferous epithelium at which these cells are formed (37) and that at which their labeling is initiated, and from the durations of these stages (25).

The duration of the preleptotene spermatocyte S-phase should be calculated by multiplying the labeling indices of these cells at stages VIII, IX, and XI by the durations of these stages. However, only the percentages of tubules containing labeled preleptotene spermatocytes have been given (25), so these were used instead in this calculation, likely resulting in an overestimate of this value.
Table 2 Suppression of spermatid production by treatment with T + E

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>No. of sonication-resistant late spermatids/testis (×10⁶)*</th>
<th>Ratio of cells in indicated region relative to 2C peakb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haploid (1C)</td>
<td>S-phase (DNA analysis)</td>
</tr>
<tr>
<td>Control</td>
<td>271 (266, 274)</td>
<td>1.59 ± 0.08</td>
</tr>
<tr>
<td>T + E, 6 wks</td>
<td>5.8 (4.8, 6.9)f</td>
<td>0.20 ± 0.03f</td>
</tr>
</tbody>
</table>

* Mean (−1 SE, +1 SE) obtained using log-transformed data.

† Mean ± SE.

‡ Significantly different from control at P < 0.001.

Methodology:

Effect of Hormone Treatment on Spermatogonial Counts. In control rats, the numbers of undifferentiated A spermatogonia per Sertoli cell increased between stages II and IV but did not significantly change further through stage VII (Fig. 1a). The numbers of differentiating cells increased from about 120 intermediate spermatogonia/100 Sertoli cells in stages III-IV to about 450 preleptotene spermatocytes in stage VII (Fig. 1b). The numbers of spermatogonia or preleptotene spermatocytes in T+E-treated rats were not significantly different from those observed in controls (P > 0.05 at all stages).

The mitotic indices of spermatogonia were measured (Fig. 1c), but divided once and twice. The number of A₄ spermatogonia was multiplied by 4 because, although they have divided three times, the ratio of green fluorescence of the most highly labeled cells/unlabeled cells was only 8, and we estimated that the progeny of the A₄ spermatogonia would have been at the limit of resolution of labeled cells. Cells earlier than the A₄ stage would have divided at least four times and contained insufficient BrdUrd to be resolved as labeled cells.

Assessment of Procarbazine-induced Damage to Spermatogenesis. Procarbazine (N-(1-methylethyl)-4-[[2-methylhydrazine]methyl]benzamide monohydrochloride) was a gift from Hoffmann-La Roche (Nutley, NJ). It was dissolved in normal saline and injected i.p. into rats (four rats/group).

Recovery of spermatogenesis from stem spermatogonia was evaluated 9 weeks after procarbazine injection. The right testis was weighed after removal of the tunica albuginea. The tissue was homogenized and sonicated as described previously (39, 40), and sperm heads (nuclei of sonication-resistant, step 12-19 elongated spermatids) were counted using a hemacytometer. The left testis was fixed in Bouin’s fluid and processed for histology.

Data Analysis. The effects of hormone treatment on spermatogenic and cell kinetic parameters were analyzed using the t test. Determination of the increases in numbers of A₄ spermatogonia through the stages was performed by linear regression.

RESULTS

Suppression of Spermatid Production. Treatment of these rats with T+E for 6 weeks resulted in the almost complete absence of late spermatids in histological sections (data not shown), as described previously (10, 27). The numbers of late spermatids in testis homogenates were reduced 50-fold (Table 2). The suppression of spermatid development was further confirmed by analysis of the flow cytometric DNA histograms; the number of early spermatids (1C DNA content) was reduced 8-fold from control levels (Table 2). In contrast, there was no significant reduction in the numbers of spermatocytes as determined from the ratio of 4C/2C cells.

Protection of Recovery of Spermatogenesis from Surviving Stem Cells. To confirm that in the current experiment the suppression of spermatogenesis with T+E for 6 weeks did protect spermatogenesis from procarbazine-induced damage, we assessed the testis weights, repopulation indices, and sperm head counts at 9 weeks after procarbazine treatment. The results (Table 3) confirm highly significant protection for all end points (10, 11).
Fig. 2. Distributions of DNA contents of testicular cells from control rats pulse-labeled with BrdUrd and killed at various times after labeling. Testis cells were subjected to flow cytometric analysis. Only the BrdUrd-labeled cells were selected, and the DNA histogram of these cells was obtained. The positions of the haploid (1C), diploid (2C), and tetraploid (4C) peaks and the S-phase cells (between 2C and 4C) are marked.

Cell Cycle Kinetics of Labeled Cells. Although the frequencies of mitotic and labeled cells were unchanged, the above data provided no information on whether the rate of progression of the cells through their cell cycles also remained the same. To measure the rates of progression, cells were harvested at different times after pulse-labeling with BrdUrd for flow cytometric analysis of the labeled cells (Fig. 2). Immediately after BrdUrd injection, the labeled cells were distributed throughout S-phase. Within 8 h, the labeled cells had progressed toward late S-phase and accumulated in the G2M phase of the cell cycle (4C); only a small fraction of labeled cells had divided and was found in the G1 (2C) region. By 16 h, nearly all of the labeled cells had left S-phase; many labeled cells were in the G2M region, but some had divided to become G1 cells. At 27 h, none of the labeled cells remained in their original S-phase; even more had divided to become G1 cells, and some of these had entered their second S-phase. At 42 h, three populations of BrdUrd-labeled cells were observed: (a) the G1 population mostly represented cells that had become preleptotene spermatocytes, which have a long G1 phase (Table 1); (b) the population with S-phase DNA content were cells that are still spermatogonia, which have 40–42-h cell cycle times; and (c) the 4C population consisted largely of primary spermatocytes in their long meiotic prophase. At 16.9 days, a peak at the 1C position was observed; it represented labeled cells that had passed through both meiotic divisions to become spermatids. In addition, a 2C peak was observed; it

because of a tendency for mitotic cells to appear in clusters, there was a large degree of variability, especially in stages II and III, in which the numbers of spermatogonia were low. Nevertheless, we found significant levels of mitoses in stages IV and VI, corresponding to divisions of intermediate and B spermatogonia, respectively, and very low levels of mitoses in stages V and VII. Again, there were no significant differences in this parameter between control and T+E-treated rats (P > 0.15 at all stages). Thus, the intermediate and B spermatogonia seem to be proliferating in the hormonally suppressed testes.

Effect of Hormone Treatment on S-Phase Cell Numbers. To further demonstrate that these cells were proliferating in the hormonetreated rats, we determined the numbers of S-phase cells by flow cytometry. The 2C peak was used for normalization because it is composed of primarily of nongerminal cells and G1-phase spermatogonia, which are not lost as a result of hormone treatment. Analysis of the one-dimensional DNA histograms of testis cells showed that hormonal treatment produced no significant change in the numbers of S-phase cells, which were 27–29% of the numbers of 2C cells (Table 2). Because S-phase fractions from one-dimensional DNA histograms may be subject to errors as a result of background debris or clumps of cells in this region and imprecisions involved in computation (41), the percentage of S-phase cells was determined more precisely from bivariate BrdUrd-DNA histograms. By this method, the ratio of S-phase cells:2C cells was 18% in both control rats and those in which sperm production was hormonally suppressed. Thus, the numbers of proliferating spermatogonia and preleptotene spermatocytes are unchanged by the hormone treatment.
corresponded only to secondary spermatocytes because almost no spermatogonia remained labeled at this time.

The kinetics of spermatogonia and preleptotene spermatocytes progression were quantified by parameters presented in Fig. 3. The theoretical curves calculated from published autoradiographic data on spermatogonial kinetics in control rats (26, 37) agreed quite well with the experimental values for RM and I determined by flow cytometry (Fig. 3, a and b). There seemed to be a slight discrepancy between the theoretical curves for I and the experimental ones (Fig. 3c). At 16 and 27 h, the experimental values of I were slightly lower than the theoretical one, probably because some of the cells that entered the second S-phase had already progressed to the second G2 or M phase and were not included in I.

Comparison of T+E-treated rats with control rats shows that the kinetics of progression of spermatogonia and preleptotene spermatocytes through their cell cycles are remarkably similar in both groups of rats. There were, however, subtle differences. The curves for RM actually indicated roughly a 20% faster progression of cells through S-phase in the treated rats than in the control rats (Fig. 3a). Because the preleptotene spermatocytes contribute most to the RM curve (Table 1), this result most likely represents a shortening of the S-phase duration of preleptotene spermatocytes. The values for I and I reflect the progression of cells labeled as spermatogonia because cells labeled as preleptotene spermatocytes do not divide until much later. There were no significant differences in the progression of spermatogonia between control and T+E-treated rats. Although a greater fraction of labeled cells seemed to have divided in the control animals than in the T+E-treated animals at 42 h after BrdUrd injection, these data are imprecise because some of the divided cells had already passed into their G2 or M phase and were not counted.

The passage of labeled cells through the meiotic divisions (Fig. 4) also shows that the labeled preleptotene spermatocytes progress through meiotic prophase in hormone-treated rats. At 406 h, a significantly greater number of labeled cells have completed the second meiotic division in the T+E-treated rats than in the control rats (Fig. 4b), indicating that hormone treatment increased the rate of progression through meiotic prophase. The labeled cells seemed to be 3–5 h more advanced in the completion of meiotic prophase in the T+E-treated rats than in the control rats; this represents only a 1% decrease in the duration of meiotic prophase in the hormone-treated rats.
HORMONAL PROTECTION OF SPERMATOGENESIS

DISCUSSION

Spermatogonial Numbers and Kinetics. This study conclusively shows for the first time that spermatogonial numbers and kinetics are not affected during hormone treatments that protect the recovery of spermatogenesis from cytotoxic agents such as procarbazine and irradiation (18). Thus, the mechanism originally proposed by Glode et al. (19), that hormone treatment might protect the testis from cytotoxic damage by reducing the rate of spermatogenesis, cannot be correct. Furthermore, an alternative mechanism proposed by van Alphen et al. (21) for FSH-induced protection of monkey spermatogenesis, that spermatogonial numbers are increased and that their proliferation is stimulated, does not apply to protection in the rat in which sperm production is suppressed with gonadal steroids analogues.

The BrdUrd labeling showed that the percentage of cells in S-phase and their subsequent kinetics were essentially unaffected by hormone treatment. Because most of the BrdUrd-labeled cells are preleptotene spermatocytes, B spermatogonia, and intermediate spermatogonia, the kinetic information applies directly to those cells. Although no marked effects of hormone treatment were observed, there were some slight but statistically significant kinetic differences between the control and hormone-treated rats. There was a significant reduction in the duration of S-phase of preleptotene spermatocytes in the hormone-treated rats. There was also a 1% decrease in the time for the most advanced labeled preleptotene spermatocytes to pass through their meiotic divisions. These differences are small, and the mechanisms responsible for their induction and their biological significance are both unknown; in any case, they indicate a slightly more rapid development of spermatocytes in the rats in which sperm production was suppressed.

Because the numbers and kinetics of intermediate and B spermatogonia are unchanged by hormone treatment, their production from A spermatogonia must also be unaffected. The A spermatogonia must therefore be proliferating, or else their numbers would be depleted by the production of intermediate spermatogonia. Although we have not examined the A₁ through A₄ spermatogonia, which are found in stages VIII through I of the seminiferous epithelial cycle, we counted their precursors, the A-aligned spermatogonia in stages II to VII (Fig. 1a). The absolute numbers of these cells, as well as the increase in their numbers between stages II and VII of the cycle (35), were unaffected by hormone treatment. This indicates that the A-aligned spermatogonia must have been proliferating because otherwise their numbers would have been depleted by the production of A₁ spermatogonia. If these cells progress through their differentiation pathway, their precursors, the A-isolated stem spermatogonia, must also proliferate. Although we cannot directly measure the kinetics of the stem cells, there is no reason to believe, because their progeny were present in normal numbers and were proliferating at a normal rate, that their numbers or rate of proliferation changed. It should be noted that a large increase in the number of stem cells would be required to account for the 9-fold increase in repopulation indices and the 600-fold increase in recovered level of sperm counts in hormone-treated rats after procarbazine injection. Thus, alterations of spermatogonial numbers or kinetics cannot be responsible for the protection observed.

Implications for Mechanisms of Protection. Alternate mechanisms by which hormone pretreatment protects the ability of spermatogenesis to recover after cytotoxic damage must be considered. The possibility that alterations in drug bioactivation, distribution to tissues, and systemic drug detoxification account for the protection was ruled out by studies showing that the protection against procarbazine was specific for the testicular recovery from surviving stem cells; other tissues or other germ cells were not protected (11). Other possibilities considered were that the hormone treatment induced resistance in the target cells either by the: (a) elevation of thiols, resulting in detoxification of active drug metabolites or scavenging of radicals; (b) enhancement of DNA damage repair; or (c) decrease in tissue oxygen levels, resulting in lower local bioactivation of chemotherapeutic drugs or decreased fixation of radical damage on DNA. However, these possibilities are inconsistent with the observation that hormone treatment protects against neutron irradiation² as well as it did against γ-irradiation. This is because neither DNA repair, free radical scavenging, nor reversal of DNA radicals affects the survival of target cells exposed to high linear energy transfer radiations, such as neutron radiation.

It may be that hormone pretreatment does not actually protect the stem spermatogonia from damage by cytotoxic treatment nor enhance their survival. Alternatively, we suggest that hormone pretreatment protects spermatogenesis primarily by enhancing its recovery from the surviving stem cells after the cytotoxic insult by the reduction of intratesticular testosterone levels caused by the T+E treatment (27). Indirect support for this hypothesis is obtained from observations that spermatogenesis often fails to recover after cytotoxic insult despite the presence of surviving stem spermatogonia (42), but recovery can be stimulated by suppression of intratesticular testosterone levels after the cytotoxic treatments (43). The possibility that suppression of intratesticular testosterone before cytotoxic treatment is stimulating recovery by the same mechanism is under investigation.

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² Unpublished observations.
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