Absence of Testicular Protection by a Gonadotropin-releasing Hormone Analogue against Cyclophosphamide-induced Testicular Cytotoxicity in the Mouse

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ABSTRACT

Protection of testicular integrity against damage from cyclophosphamide (CY) by simultaneous treatment with a gonadotropin-releasing hormone (GnRH) analogue was reported in BALB/c mice (L. M. Glode et al., Lancet, 1: 1132–1134, 1981). This approach has been used as the basis for clinical trials in various treatment centers (D. H. Johnson et al., Blood, 65: 832–836, 1985) in an attempt to prevent iatrogenic sterility toward protecting spermatogenesis is to inhibit the rapid cycling of stem cells that appears to occur during recovery from cytotoxic insults. It has been suggested that this inhibition could be accomplished by suppression of testosterone production through manipulation of the pituitary-gonadal axis, as the process of spermatogenesis depends upon testosterone. The major alteration in spermatogenesis caused by the absence of sufficient androgen stimulation appears to be the death of cells as they develop through the spermatocyte and spermatid stages; it is worthy of note that no alterations of kinetics of spermatogenesis are produced by these hormonal changes.

One approach to endocrine-mediated inhibition of spermatogenesis has been continuous administration of analogues of LHRH or GnRH. This treatment results in a reduction of LH release, testosterone production, and inhibition of spermatogenesis in the rat (5), dog (6), monkey (7, 8), and humans (9). Glode et al. (10) proposed that such inhibition of spermatogenesis by GnRH could protect spermatogenesis in the mouse against the cytotoxic action of CY by placing the testes in a "quiescent" state. In that study (10), clinical observations of reduced damage by cytotoxic drugs on the prepubertal (i.e., quiescent) testes were quoted in support of that mechanism.

Publication of Glode's study (10) on the possibility of gonadal protection (11) has stimulated the initiation of extensive investigations in large experimental animals (7, 8), and as a basis for the implementation of clinical trials (12). Because of the consequences of that work, further clarification and extension of that brief report would be very important. In particular, three points need to be considered. (a) According to the proposed mechanism of action, GnRH must inhibit spermatogenesis and stem cell proliferation in order to produce protection. No inhibition of spermatogenesis and hence stem cell proliferation was observed in that study nor were alternative mechanisms of GnRH action offered. In this study we test the possibility of inhibition of spermatogenesis by GnRH by quantitative assessment of spermatogenesis in mice treated with GnRH alone. We have included assays for stem cell killing because this event is responsible for the long-term treatment-induced sterility, and therefore, the major source of concern to cancer patients (13, 14). (b) The previous assessment of CY toxicity was heavily based on observations of tubular disorganization, a subjective end point whose variations can sometimes be ascribed to fixation artifacts. Our present findings are based on quantitative assays (15) and parallel histological analysis. We believe that quantitative end points are essential in defining damage by, or protection against, cytotoxic damage. (c) In the previous study
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(10), data were obtained from (histological) analysis of only 6 mice, 2 in the CY + GnRH group, and 4 in the CY-only group. We have increased the number of experimental animals to provide for more adequate statistical analysis of quantitative end points.

In summary, we have attempted here to repeat the protocol of the previous study (10) and to try to answer, with various quantitative end points, the questions that were unanswered in that study. We report on the characterization of the time course of damage to the seminiferous epithelium, following CY treatment, as a necessary base-line value for studies of protection; the quantitation of CY-induced testicular damage by testicular weight measurements and sperm head counts; and the use of assay times that will yield data on stem cell survival.

MATERIALS AND METHODS

Mice and Drugs. Male BALB/c mice, 12 wk old at the start of these experiments, were purchased from Harlan Sprague-Dawley, Incorporated, Houston, TX. The animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and in accordance with current regulation and standards of the United States Department of Agriculture and the NIH, Department of Health and Human Services.

GnRH (o-leucine-6 LHRH) was obtained through the courtesy of Abbot Laboratories, North Chicago, IL. CY was produced by Mead-Johnson Pharmaceutical Division, Evansville, IN.

Experimental Design. These studies were planned to replicate the experimental design used by Glode et al. (10). The only modification introduced in the treatment schedule was to increase the exposure time to GnRH from 22 to 23 days. This was done to ensure that the circulating level of GnRH would be maintained for at least 24 h past the last CY injection.

Group A, or control group (n = 20), were given 23 daily i.p. injections of 0.2 ml of normal PBS plus 0.2 mg of BSA. Group B mice (n = 20) were given 23 daily s.c. injections of GnRH (0.2 ml of PBS containing 0.4 µg GnRH plus 0.2 mg of BSA). Group C mice (n = 40) were given 23 daily s.c. injections of GnRH, as above, and in addition, on Days 8, 15, and 22, they were given i.p. injections of CY (200 mg/kg). Group D mice (n = 40) were given CY only on Days 8, 15, and 22, as above.

Animal Survival. The number of mice that survived treatment and were available for assay, as compared to those at the onset of the experiment, were as follows: Group A, 19 of 20 (95%); Group B, 20 of 20 (100%); Group C, 28 of 40 (70%); Group D, 35 of 40 (88%).

Statistical Analysis. The sperm head counts, presented in Fig. 3, were calculated as algebraic means of counts from individual mice at each assay point. The significance in the difference between control and treated groups in all assays (including testes weights) was determined by a 2-tailed t test.

RESULTS

Qualitative Analysis of the Effects of CY on Spermatogenesis. The first step in this study was to determine, as a base-line value, the characteristic changes that occur in the testes following CY treatment (Fig. 1). Histological analysis of seminiferous tubules from a control mouse (Stage V or VI) reveals the presence of type B spermatagonia, midpolygonal spermatocytes, round spermatids, and elongated spermatids in an orderly series of layers (Fig. 1a). The most prominent feature observed upon histological analysis of testicular cross-sections from mice treated with CY was the absence of specific germ cell types. On Day 2 (3, 10, 17 days following each CY injection), essentially all tubules showed the absence of spermatagonia (past the type A stage) and spermatocytes. Elongated spermatids were present in all tubules, and approximately 50% of the tubules also contained round spermatids, as shown in Fig. 1b. The presence of these cell types indicates the resistance of primary spermatocytes and all classes of spermatids to killing by CY. On Day 14 (15, 22, and 29 days following each CY injection), some tubules contained elongated spermatids, and recovery of spermatogenesis from surviving stem cells was indicated by the presence of spermatogonia in all tubules and of primary spermatocytes in approximately half of the tubules. The Stage V-VI tubule shown in Fig. 1c contains type B spermatogonia and elongated spermatids in an orderly arrangement. The elongated spermatids represent cells that were in the type B spermatogonia and later stages at the times of treatment and hence were relatively resistant to cytotoxicity. On Day 29 (30, 37, and 44 days following each CY injection), regeneration of tubules was performed with a hemacytometer under phase-contrast microscopy. This time point corresponds to 30, 28, and 44 days after the CY injections. The number of sperm heads at this time is a measure of survival of stem plus differentiating spermatogonia (15). On Day 56 after cessation of GnRH treatment, animals available as above from each group were killed, and testes were processed for histological analysis. Quantitative measurement of elongated spermatids was performed, in this case, from the histological sections. Four mice were studied in each of the Groups A, B, and C, and 8 in Group D. The germ cells in the seminiferous epithelium of most mammals are arranged in consistent patterns of cellular associations that define, in the mouse, 12 developmental stages referred to as the stages of the cycle of the seminiferous epithelium (17). Precise identification of each stage can be established by the morphology of the differentiating spermatids in a sequence that begins with the early round spermatids (Stage I) and progresses through the elongation of the spermatid to assume a shape similar to that of the mature sperm (Stage XII). Later forms of elongated spermatids are found in tubules of Stages I to VIII in association with round spermatids. From the stained testicular cross-sections obtained from each mouse, 15 early stage tubules (Stages I to III) were selected, and the numerical ratio of elongated spermatids to Sertoli cells was established. Since Sertoli cells are not sensitive to the doses of CY used, and since they do not divide in the mature testis, this stable population of cells is commonly used as a normalizing factor when counting germinal epithelium cells.

Early stages were chosen to ensure that the ratios obtained expressed the real number of spermatids present and were not altered by their release from the tubules, which occurs during Stage VIII. This is a quantitative method for measuring the survival of stem spermatogonia, since all elongated spermatids present at 57 days after the last CY injection must have arisen from cells that were stem spermatogonia during all of the CY treatments.

Statistical Analysis. The sperm head counts, presented in Fig. 3, were calculated as algebraic means of counts from individual mice at each assay point. The significance in the difference between control and treated groups in all assays (including testes weights) was determined by a 2-tailed t test.
observed with the most advanced cell type being round spermatids in one-third of the tubules (Fig. 1d), pachytene spermatocytes in over half, and in a few cases only spermatogonia or early primary spermatocytes. On Day 56 (57 to 71 days after CY injection), a qualitative (but not quantitative) recovery of spermatogenesis, with all germ cell types represented in nearly all tubules, was observed (Fig. 1e).

Only germ cell studies were included in the scope of this investigation. However, in the process of scoring for germ cell abnormalities, interstitial and Sertoli cells were carefully observed. No gross morphological or quantitative changes were seen as a result of GnRH or CY treatment in either Leydig or Sertoli cell populations.

Effects of CY and GnRH on Testicular Weight. No significant changes were observed in any of the assay points between mice in Groups A (control) and B (GnRH only) (Fig. 2). Reductions in testicular weight were observed in Groups C (GnRH plus CY) and D (CY). On Day 14, testes weight of Groups C and D animals were 40% and 46% of control weights, respectively. On Day 29, Group C and Group D mice had testicular weights...
control and experimental mice on Days 29 and 56 after completion of GnRH with and without GnRH (P < 0.03, 2-tailed t test). Number of mice assayed as times. Either 5 or 10 animals were used per point (see text). Error bars were omitted for clarity. * points significantly different between comparable groups, elongated spermatid to Sertoli cells in Group A averaged 14.8 ± 0.7 (range, 14.1 to 17.0). For Group B, the average ratio was 15.4 ± 0.7, and the range was 14.1 to 17.0. Sperm counts in both Groups C and D at this same time point were reduced to 40% of control levels, indicating partial killing of differentiating spermatogonia. The ratio of elongated spermatids to Sertoli cells in Group C averaged 6.3 ± 1.6 (range, 1.6 to 9.1), and in Group D the average ratio was 6.3 ± 0.2, with a range of 5.7 to 7.4. There were no significant differences between Groups C and D.

**DISCUSSION**

The aim of this investigation was to replicate the work of Glode et al. (10), who had reported that administration of GnRH offered testicular protection against cytotoxic damage by CY. We felt that a more detailed study, based on quantitative end points, was necessary if that approach was to be used as justification for clinical trials with cancer patients facing cytotoxic treatment.

The results of this study emphasize the importance of obtaining a reliable base-line value for damage by a chemotherapeutic agent and a means of quantitative assessment of that damage before attempting to study the possible effects of protective treatments. Disorganization of the seminiferous epithelium should not be used as the only reliable end point, and it should only be used in addition to quantitative measurements, such as testis weights and particularly sperm head counts, which provide objective end points amenable to statistical analysis. Our results show clearly that the effects of CY treatment can be described by killing of differentiating spermatogonia followed by an orderly maturation depletion of later germ cell stages and repopulation from surviving cells. As early as Day 2 (3, 10, and 17 days following each CY injection), the presence of round and elongated spermatids indicated the resistance of primary spermatocytes and all classes of spermatids to killing by CY. This finding is consistent with previous observations using single injections of CY into C3H mice, in which the only cells killed were type A, through type B spermatogonia and preleptotene spermatocytes (15). The killing of these spermatogonial stages by CY was confirmed by the complete absence of midpachytene spermatocytes and round spermatids noted on Day 14 (15, 22, and 29 days following each CY injection). Further evidence that the cytotoxic effects of CY were directed primarily towards differentiating and not stem spermatogonia was obtained by the recovery of spermatogenesis seen on Day 56 (57 to 71 days after CY treatment). At this time, recovery of all germ cell types in most, but not all, tubules indicates that numerous stem spermatogonia survive the cytotoxic effects of CY. Killing of large numbers of stem cells would have resulted in many tubules in which repopulation had not occurred and which would be lined only with Sertoli cells (18). Although a certain degree of architectural disorganization was occasionally seen, it probably represents technical artifacts created during sectioning or histological preparation. However, the main feature observed consistently, and the one with strongest biological significance, is the depletion of specific germ cell stages. Instances of apparent disorganization of the seminiferous epithelium are most likely secondary to germ cell depletion and do not constitute an adequate indicator of cytotoxic damage.

The rationale for protecting the testes against CY-induced injury by administration of a GnRH analogue has been that inhibition of the pituitary-gonadal axis, and the resulting suppression of spermatogenesis, would render the testes more resistant to cytotoxic agents (10, 11). Suppression of spermatogenesis by GnRH analogues has been widely investigated in rodents, dogs, monkeys, and humans. While lowering of testosterone levels and suppression of spermatogenesis by GnRH analogues have been obtained in rats (5), beagles (6), monkeys (7, 8), and humans (9), mice have been resistant to these actions of GnRH analogues (19). Our results have confirmed the ob-
servations that the testes of the BALB/c strain of mice are unaffected by GnRH. According to our experimental design, if there had been any effect of GnRH on stem cell proliferation, sperm head counts 29 days after daily administration of GnRH for 23 days would have been reduced. Also, no decrease in testicular weight was observed in animals treated with GnRH only. Thus, the mouse is not an adequate model for testing GnRH suppression of stem cell proliferation.

We have also demonstrated by three different quantitative assays that GnRH does not confer any protection against the cytotoxicity induced on mouse testis by CY. A CY-induced reduction in testicular weight was observed in mice treated with CY only and with CY plus GnRH. No significant difference was observed between these two groups, indicating that GnRH was not capable of reducing the testicular damage induced by CY; in fact, a slight enhancement of damage is suggested, for which we have currently no explanation.

Variations in testicular weight are not as sensitive an end point for quantitative determination of spermatogonial killing as are counts of testicular sperm heads (20). In addition, because of the fixed kinetics of spermatogenesis, the latter method permits the identification of the stages of spermatogenic cells at the time of cytotoxic insult. Significant killing of differentiating spermatogonia by CY was noted by sperm head counts performed on Day 29 (30, 37, and 44 days after CY treatment). No differences in sperm count were observed in the presence and in the absence of GnRH, indicating no observable effects on spermatogenesis, and no protection by GnRH against CY injury. A moderate cytotoxic effect of this alkylating agent was also seen on stem spermatogonia, as demonstrated by decreased sperm counts at 57 days after the last CY treatment. Again, no protection against this killing of stem cells was obtained by the administration of GnRH.

The results of this study open to question the original experiments, which have been the basis for further research and clinical trials on the use of endocrine suppression of spermatogenesis to protect the testes from cytotoxic damage. These findings stress the need for more extensive research on animal models in which GnRH inhibition of spermatogenesis would be more similar to that observed in humans. Clinical studies currently under way to evaluate protection of spermatogenesis from cytotoxic damage with GnRH failed to yield any positive results (12), but a final conclusion still depends on larger numbers of patients being studied.

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REFERENCES


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