Restoration of Spermatogenesis in Dibromochloropropane (DBCP)-Treated Rats by Hormone Suppression

Marvin L. Meistrich,*1 Gene Wilson,*2 Karen L. Porter,* Ilpo Huhtaniemi,†3 Gunapala Shetty,* and Gladis A. Shuttlesworth*4

*Department of Experimental Radiation Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030; †Department of Physiology, University of Turku, 20520 Turku, Finland

Received July 2, 2003; accepted September 3, 2003

The exposure of men to the nematocide dibromochloropropane (DBCP) has caused prolonged oligo- and azoospermia, which occasionally reverses spontaneously. We recently demonstrated that in testes of rats treated with a dose of DBCP sufficient to reduce the percentage of tubules producing differentiating germ cells (tubule differentiation index, TDI) to 20%, the tubules lacking differentiating cells contained type A spermatogonia. To determine whether these type A spermatogonia could be stimulated to differentiate, as had been demonstrated previously in other models of toxicant-induced sterility, we suppressed intratesticular testosterone and serum follicle stimulating hormone (FSH) levels with the GnRH agonist Lupron (leuprolide). When the GnRH agonist was given for 10 weeks starting immediately after DBCP exposure, the TDI was maintained at 94%. Even when GnRH-agonist treatment was stopped at week 10, the TDI remained between 65 and 80% 10 weeks later. Late spermatid counts averaged 10 × 10⁶ per testis for the GnRH-agonist–treated rats at week 20 compared with 1.7 × 10⁶ per testis in rats treated with only DBCP. To determine whether spermatogonial differentiation could be stimulated after the TDI had declined to below 30%, we initiated GnRH-agonist treatment 6 weeks after DBCP exposure. The GnRH treatment increased the TDI to 53% at week 16. These results indicate that, if the same principles apply to humans, suppression of testosterone may be applied to restore spermatogenesis in men rendered azoospermic by DBCP or other reproductive toxicants.

Key Words: spermatogonia; gonadotropin-releasing hormone analogs; reproductive toxicants.

Dibromochloropropane (DBCP), a nematocide developed in the 1950s, was widely used in agriculture until the late 1970s, when oligospermia and azoospermia were reported in manufacturing plant workers (Lipshultz et al., 1980; Potashnik et al., 1979; Whorton et al., 1977) and pesticide applicators (Glass et al., 1979; Slutsky et al., 1999). The manufacture and use of DBCP in the mainland United States were banned in 1979, but the chemical continued to be used in Hawaii and was exported to other countries for at least another decade. DBCP is persistent in the environment: Measurable levels were found in well water 13 years after its use (Kloos, 1996).

About half of the DBCP-exposed azoospermic men remained that way for many years (Eaton et al., 1986; Olsen et al., 1990; Potashnik and Porath, 1995), suggesting that all of the stem spermatogonia may have been killed. Others recovered sperm count, but in some cases the recovery did not occur until 3 to 6 years later (Eaton et al., 1986; Olsen et al., 1990; Potashnik, 1983). This observation indicates that stem spermatogonia were present but unable to differentiate into spermatozoa for an extended period of time.

To develop methods for understanding and possibly reversing sterility in these men, we recently developed an animal model in which rats showed prolonged effects of DBCP on the germinal epithelium (Meistrich et al., 2003). Although spermatogenesis did not recover 20 weeks after DBCP exposure, the presence of type A spermatogonia in the tubules after DBCP treatment of these rats suggests that spermatogenesis could be restored.

The continued presence of type A spermatogonia that proliferate but die by apoptosis just prior to differentiation is a more general phenomenon (Meistrich and Shetty, 2003). For instance, it has been shown to be the case in rats that were treated with hexanedione (Boekelheide and Hall, 1991), irradiation (Kangasniemi et al., 1996), procarbazine (Meistrich et al., 1999), boric acid (Ku et al., 1993), or an indenopyridine (Hild et al., 2001) and in aging rats (Schoenfeld et al., 2001). We had also shown that GnRH-analog (agonist or antagonist) treatment given after irradiation stimulated the differentiation of these cells (Meistrich and Kangasniemi, 1997; Shuttles-
worth et al., 2000). In several of the above cases (Blanchard et al., 1998; Meistrich et al., 1999; Schoenfeld et al., 2001), as well as after testicular heating (Setchell et al., 2001) or busulfan exposure (Udagawa et al., 2001), spermatogenesis was also stimulated by the administration of a GnRH agonist after toxic exposure or the development of tubular atrophy, and in some cases fertility was restored.

Based on these observations, we tested whether a GnRH agonist administered to rats treated with DBCP could enhance spermatogenesis when given either immediately after DBCP exposure or after spermatogenesis had declined. We characterized the degree of recovery of differentiation within the seminiferous tubules during GnRH-agonist treatment. After the cessation of the hormone treatment, we also followed the maintenance of differentiation within the tubules as well as the degree of recovery of sperm production.

**MATERIALS AND METHODS**

**Rats.** Male LBNF and female Sprague-Dawley rats were obtained at 200–224 g body weight and at 7 weeks of age, respectively, from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were maintained in facilities approved by the American Association for the Accreditation of Laboratory Animal Care in accordance with current regulations and standards. The animals were kept for 1 week so they could acclimatize before the experiments were initiated.

**DBCP treatment.** Lots LA82202 and LA85851 of DBCP, purity 97–98%, were obtained from Supelco Separation Technology (Bellefonte, PA). DBCP was dissolved in corn oil and given as four daily subcutaneous (sc) injections of 87.5 mg/kg body weight.

**GnRH agonist treatment.** The GnRH agonist leuprolide acetate, ([D-Leu⁶, des-Gly-NH₂⁰, Pro-ethylamide⁹]GnRH), was supplied as a nominal one-month depot formulation (Lupron) in microspheres of a lactic acid–glycolic acid copolymer by TAP Pharmaceuticals (Deerfield, IL). Leuprolide was suspended in a carboxymethyl cellulose vehicle provided by the manufacturer and given as 1.83-mg intramuscular (im) injections 23–24 days apart. This timing was chosen based on data showing that the drug is released over a 4-week period with maintenance of serum leuprolide levels above 2 ng/ml and suppression of serum testosterone levels for that period of time (Ogawa et al., 1989). Serum testosterone was suppressed starting at 3 days after starting treatment (Ogawa et al., 1989), and we have shown with a different GnRH agonist that follicle stimulating hormone (FSH) and intratesticular testosterone levels reached minimal values within 4 days (Kangasniemi et al., 1995).

Two different treatment schedules were used. To study the maintenance of spermatogenesis after DBCP treatment (Experiments 1 and 2), the leuprolide treatment was started 1 day after the last DBCP injection, and a total of three injections, 23–24 days apart, were given to provide exposure to the hormone for about 10 weeks (Fig. 1). Groups for comparisons and controls consisted of untreated rats, rats treated with DBCP alone, and rats treated with leuprolide only. Some rats were killed at week 10, near the end of the GnRH-agonist treatment, and others were killed at week 20 to assess whether spermatogenesis was still maintained after GnRH-agonist treatment was suspended or whether it regressed to the levels measured in DBCP-only–treated rats. To study the restoration of spermatogenesis after it had declined (Experiments 3 and 4), leuprolide treatment was started 6 weeks after the last DBCP injection, and injections were given 23–24 days apart until the rats were killed, 5.6 (Experiment 3) or 10 weeks later (Experiment 4). Because there was some variability in the effectiveness of DBCP between experiments (Meistrich et al., 2003), results for some of the endpoints were plotted separately.

**Evaluation of recovery of spermatogenesis and fertility.** In all of the experiments, when the rats were killed, half of the left testis was fixed in Bouin’s fluid and the other half was fixed in 70% ethanol at 4°C. Tissues were embedded in methylacrylate, and sections were stained with Harris hematoxylin. Tubules showing other forms of damage in addition to just the lack of germ...
cells, such as occlusion, flattened Sertoli cells, or the absence of Sertoli cells (Meistrich et al., 2003), were counted. Tubules without such damage were then scored to determine the tubule differentiation index (TDI). The TDI is the percentage of tubules that contained three or more differentiated spermatogenic cells more advanced than type A spermatogonia (i.e., intermediate or type B spermatogonia, spermatocytes, or spermatids) and is a measure of survival and differentiation of the stem, type A spermatogonia (Meistrich and van Beek, 1993). One transverse section was taken from the center of one testis from each rat, and at least 200 seminiferous tubule cross sections were scored in that section to determine the TDI. When counts performed 6 weeks after DBCP administration, cells beyond step 1 round spermatids were excluded from the counts, since these cells would have derived from cells that were already differentiating at the time of DBCP treatment (Kangasniemi et al., 1996).

The right testis was weighed after the removal of the tunica albuginea, and the tissue was then homogenized. Approximately 1 ml of the testis homogenate was removed from the rats killed at week 20 in Experiments 1 and 2 and sonicated (Meistrich and van Beek, 1993), and an aliquot was placed in a hemacytometer to count late spermatid nuclei.

To assess fertility, male rats in Experiments 1 and 2 were mated for 2 weeks before they were killed at week 20. Each male was caged with two females for 1 week, and then the females were replaced with two different females. The females were killed 14 days after the end of mating, and the embryos were counted. A male was considered fertile if he produced one or more offspring by any of the four females.

**Characterization of type A spermatogonia.** Type A spermatogonial cells, mitoses, and cells with apopotic morphology were counted in 200 atrophic seminiferous tubule cross sections, without additional morphological abnormalities, from the Bouin’s fixed tissue (Shuttlesworth et al., 2000). Type A spermatogonia were identified by their round or ovoid nuclei with fine granular chromatin and one to three nucleoli that are sometimes adjacent to the nuclear periphery (Chiarini-Garcia and Russell, 2001). To normalize the count of type A spermatogonia to the numbers of Sertoli cells, the Sertoli cells with visible nucleoli were counted in 20 (every tenth) seminiferous tubular cross sections from each rat. The diameters of type A spermatogonia, mitoses, apoptotic cells, and Sertoli cell nucleoli previously measured were used to calculate the relative volume densities of the cells using the Abercrombie correction (Shuttlesworth et al., 2000).

To determine the labeling index of type A spermatogonia, the rats were injected i.p. with bromodeoxyuridine (BrdU) at 30 mg/kg, 1 h prior to killing. The ethanol-fixed testicular tissue was processed for BrdU immunohistochemistry using protease digestion, clone B44 first antibody (Becton Dickinson Immunocytometric Systems, San Jose, CA), and the Vectastain kit (Vector Laboratories Inc., Burlingame, CA) as described in Shuttlesworth et al. (2000). Type A spermatogonia were counted in 200 seminiferous tubular cross sections to determine the labeling index.

**Hormone measurements.** From some of the rats, blood was collected at the time they were killed by cardiac puncture under ketamine–acepromazine anesthesia. The serum was separated and stored at ~80°C.

The right testis was homogenized and stored at ~80°C for analysis of intratesticular testosterone (ITT). Serum testosterone and ITT were assayed using coated tubes (DSL 4000, Diagnostic Systems Laboratories, Webster, TX) (Shetty et al., 2000). ITT was expressed as the amount per gram of testis rather than the amount per testis to reflect the concentration of testosterone to which the testicular cells were exposed. The minimum levels of detection for serum testosterone and ITT were 0.04 ng/ml and about 0.6 ng/g-testis, respectively.

The serum levels of FSH and luteinizing hormone (LH) were measured using immunofluorometric assays (Delfia, Wallac OY, Turku, Finland) as described in Haavisto et al. (1993) and van Casteren et al. (2000). The minimum levels of detection of LH and FSH by this method were 0.04 ng/ml and 0.1 ng/ml, respectively.

**Statistics.** All data are presented as the mean ±1 SEM using either the untransformed data or log-transformed data for plots on a logarithmic scale.

Pairwise comparisons between groups were done using the Student’s t-test. Comparisons involving multiple groups were done by first performing a one-way ANOVA and proceeding if there were significant differences between groups (P < 0.05). A single group or multiple groups were compared to a single control using the Student’s t-test or Dunnett’s test, respectively. The relationship between fertility and the testicular characteristics was tested by Spearman’s rho, which is a measure rank order. All analyses were done using SPSS software (version 10.0, SPSS Inc., Chicago, IL).

**RESULTS**

**Effects of GnRH Agonist Treatment on Hormone Levels**

Since we treated the rats with GnRH agonist after DBCP exposure and we had previously shown that DBCP treatment increased FSH, LH, and ITT levels (Meistrich et al., 2003), the effects of the GnRH-agonist treatment on these hormone levels were established. Treatment with GnRH agonist, started immediately after DBCP exposure or 6 weeks later, significantly reduced FSH, from 8 to 10 ng/ml in DBCP-treated rats to between 1 and 2 ng/ml (Fig. 2a). In contrast, the GnRH agonist did not reduce LH levels, either in the controls or DBCP-treated rats, but produced slight, statistically significant increases (Fig. 2b), as reported in Meistrich et al. (2001) and Shetty et al. (2000). Despite the failure of the GnRH agonist to reduce LH levels, it was effective at reducing both serum and intratesticular testosterone levels (Figs 2c and 2d) presumably by direct inhibitory action on the Leydig cells (Bambino et al., 1980). When GnRH-agonist treatment was given immediately after DBCP injections, testosterone levels were reduced to levels seen in the GnRH-agonist-only–treated rats; but when the GnRH-agonist treatment was initiated 6 weeks later, the suppression of testosterone was not as effective. The suppressive effects of the GnRH-agonist treatment on the hormone levels completely reversed within 10 weeks after cessation of treatment. In both the controls and DBCP-only–treated animals, the hormone levels at week 20 were not significantly different from those in rats that had not been treated with GnRH agonist.

**Maintenance of Spermatogenesis with Immediate GnRH Treatment**

When the GnRH agonist was given for 10 weeks starting immediately after DBCP treatment (Experiment 1), the TDI was maintained at 94%, as opposed to declining to 36% in the rats not treated with the GnRH agonist (Fig. 3). Even though the GnRH-agonist exposure ended at about week 10, the TDI remained at 65% (Experiment 1) or 82% (Experiment 2) at week 20. In all groups, 1 to 15% of the tubules were abnormal with occlusion, flattened epithelium, or lack of Sertoli cells. Neither the GnRH-agonist treatment nor the time after DBCP exposure showed any consistent effect on the incidence of these tubular abnormalities

To determine if this repopulation resulted in the formation of mature gametes, late spermatid counts were performed when

**Comparisons involving multiple groups were done by first performing a one-way ANOVA and proceeding if there were significant differences between groups (P < 0.05). A single group or multiple groups were compared to a single control using the Student’s t-test or Dunnett’s test, respectively. The relationship between fertility and the testicular characteristics was tested by Spearman’s rho, which is a measure rank order. All analyses were done using SPSS software (version 10.0, SPSS Inc., Chicago, IL).**

**RESULTS**

**Effects of GnRH Agonist Treatment on Hormone Levels**

Since we treated the rats with GnRH agonist after DBCP exposure and we had previously shown that DBCP treatment increased FSH, LH, and ITT levels (Meistrich et al., 2003), the effects of the GnRH-agonist treatment on these hormone levels were established. Treatment with GnRH agonist, started immediately after DBCP exposure or 6 weeks later, significantly reduced FSH, from 8 to 10 ng/ml in DBCP-treated rats to between 1 and 2 ng/ml (Fig. 2a). In contrast, the GnRH agonist did not reduce LH levels, either in the controls or DBCP-treated rats, but produced slight, statistically significant increases (Fig. 2b), as reported in Meistrich et al. (2001) and Shetty et al. (2000). Despite the failure of the GnRH agonist to reduce LH levels, it was effective at reducing both serum and intratesticular testosterone levels (Figs 2c and 2d) presumably by direct inhibitory action on the Leydig cells (Bambino et al., 1980). When GnRH-agonist treatment was given immediately after DBCP injections, testosterone levels were reduced to levels seen in the GnRH-agonist-only–treated rats; but when the GnRH-agonist treatment was initiated 6 weeks later, the suppression of testosterone was not as effective. The suppressive effects of the GnRH-agonist treatment on the hormone levels completely reversed within 10 weeks after cessation of treatment. In both the controls and DBCP-only–treated animals, the hormone levels at week 20 were not significantly different from those in rats that had not been treated with GnRH agonist.

**Maintenance of Spermatogenesis with Immediate GnRH Treatment**

When the GnRH agonist was given for 10 weeks starting immediately after DBCP treatment (Experiment 1), the TDI was maintained at 94%, as opposed to declining to 36% in the rats not treated with the GnRH agonist (Fig. 3). Even though the GnRH-agonist exposure ended at about week 10, the TDI remained at 65% (Experiment 1) or 82% (Experiment 2) at week 20. In all groups, 1 to 15% of the tubules were abnormal with occlusion, flattened epithelium, or lack of Sertoli cells. Neither the GnRH-agonist treatment nor the time after DBCP exposure showed any consistent effect on the incidence of these tubular abnormalities

To determine if this repopulation resulted in the formation of mature gametes, late spermatid counts were performed when
the DBCP-treated rats were killed at week 20, about 10 weeks after the cessation of the GnRH treatment. The effects of the GnRH-agonist treatment alone were partially reversible, as indicated by the return of testis weights and late spermatid counts to 88 and 31% of the control values, respectively (Figs 4a and 4b). In the DBCP-treated rats, the GnRH-agonist treatment markedly increased late spermatid counts, from 4.1 × 10⁶ to 12.2 × 10⁶ per testis in Experiment 1 and from 1.0 × 10⁶ to 8.4 × 10⁶ in Experiment 2 (P < 0.001). There was a positive correlation between the TDI and sperm counts on a rat-by-rat basis in most cases, but it only reached significance in a few groups. The lack of a better correlation was due in part to the fact that the incidence of abnormal tubules varied slightly between animals and groups, and such tubules were excluded from the denominator for the calculation of the TDI.

Experiment 1 indicated a trend (P = 0.09) toward increased fertility of the GnRH-agonist–treated rats (Fig. 4c). In Experiment 2, the DBCP treatment had stronger effects, and, although the late spermatid counts were enhanced by the GnRH-agonist treatment, the levels were just below the threshold required for fertility (Fig. 5). However, one of the DBCP-only–treated rats, which was an outlier with a TDI of 85%, a testis

FIG. 2. Serum FSH (a), serum LH (b), serum testosterone (c), and intratesticular testosterone (ITT) (d) concentrations. Control rats (no treatment) and those treated with DBCP only are indicated by open bars. Rats treated with the GnRH-agonist leuprolide and killed while still on GnRH-agonist treatment are indicated by filled bars. Rats treated with GnRH agonist and killed 10 weeks after the end of the GnRH-agonist treatment are indicated by the shaded bars. The duration of the GnRH-agonist treatment is indicated below the bars for the rats not treated with DBCP. The weeks after DBCP treatment that the GnRH-agonist exposure was initiated and ceased are indicated below the bars for the rats treated with DBCP. The different open bars for DBCP-only rats represent rats treated and killed at the same time as the corresponding group also treated with GnRH agonist in the adjacent filled or shaded bar. Note the FSH values are plotted on a linear scale whereas the others are plotted on a logarithmic scale. n = 3–14 rats per point for those not treated with DBCP and n = 6–21 rats per point for those treated with DBCP. *, **, ***Significantly different from corresponding group not treated with GnRH agonist, P < 0.05 or < 0.01, respectively (Dunnett’s or t-test). #, ###Significantly different from corresponding group treated with GnRH agonist but killed at the end of GnRH treatment instead of allowing a 10-week period for reversal of hormonal suppression, P < 0.05 or < 0.001, respectively (t-test). §, §§§Significantly different from level when a 10-week GnRH-agonist treatment was started immediately after DBCP administration, P < 0.05 or < 0.001, respectively (t-test).
weight of 1.04 g, and a late spermatid count of $1.6 \times 10^8$, was fertile.

Fertility depended on testicular characteristics and was positively correlated with late spermatid counts and percentage of repopulating tubules (using total tubules as the denominator) and negatively correlated with the percentage of abnormal tubules. The highest correlation coefficient (Spearman’s rho correlation coefficient = 0.48, $P = 0.001$) was with late spermatid counts. All six DBCP-treated rats (with or without GnRH agonist) with counts above $2 \times 10^7$ were fertile, and 35/36 rats with late spermatid counts below that level were sterile (Fig. 5). Furthermore, calculation of a “fertility index,” which is the product of the percentage of males that are fertile, the percentage of females that were pregnant, and the number of embryos per pregnant female, was correlated with the late spermatid counts for the seven fertile rats (Spearman’s rho correlation coefficient = 0.75, $P = 0.05$).

**Restimulation of Spermatogenesis with Delayed GnRH Treatment**

To determine whether spermatogonial differentiation could be stimulated after the TDI had declined, we initiated GnRH-agonist treatment 6 weeks after DBCP exposure. At this time the TDIs had already declined to below 30% (Fig. 6).

In Experiment 3, 5.6 weeks of GnRH-agonist treatment increased the TDI from 25 to 47%. In Experiment 4, a 10-week treatment increased the TDI from 12 to 52%. Since the TDI remained constant with time in the rats not given GnRH-agonist treatment, these results demonstrate that the hormonal treatment restimulated spermatogenesis after DBCP-induced toxicity.

In these two experiments, the incidence of abnormal tubules was between 5 and 19% in all DBCP-treated groups. The GnRH-agonist treatment did not induce any significant changes in the incidence of these tubular abnormalities.

**Characterization of Type A Spermatogonia in GnRH-Agonist-Treated Rats**

The type A spermatogonia and Sertoli cells were counted in tubules in which no differentiating cells were observed. Surprisingly, the GnRH treatment appeared to produce a small but
significant decline in the numbers of Sertoli cells per tubule cross section (Table 1). However, stereological methods would be required to demonstrate an actual decrease in numbers of Sertoli cells per testsis.

The type A spermatogonia were rapidly proliferating, as indicated by the high labeling and mitotic indices, but instead of differentiating they were lost by apoptosis. In these atrophic tubules, the GnRH treatment slightly increased the numbers of type A spermatogonia and slightly decreased the apoptotic indices, but these changes were not statistically significant. Furthermore, GnRH-agonist treatment did not induce any observable changes in the proliferation of the type A spermatogonial population.

**DISCUSSION**

This study shows conclusively that the recovery of spermatogenesis can be stimulated in rats rendered severely oligospermic by exposure to DBCP, an important environmental and occupational toxicant. Although previous studies showed that GnRH analogs can stimulate recovery of spermatogenesis in rats with a variety of pathological situations, including aging, heating, and exposure to chemicals such as hexanedione, indenopyridine, and anticancer agents, radiation, procarbazine, and busulfan (Meistrich and Shetty, 2003), the applicability of this phenomenon to other toxicants or pathological situations was not known. Hence, it was important to test whether this stimulation of recovery similarly applied to DBCP exposure.

Qualitatively, the ability of GnRH analogs to enhance spermatogenesis after DBCP-induced toxicity was similar to the case of other toxicants. Since the studies with radiation and procarbazine were done in this laboratory with the same rat strain, we can more quantitatively compare the degrees of stimulation of recovery (Table 2) for what we estimated to be equitoxic doses of the agents based on the TD5s without GnRH-agonist treatment. The degree of stimulation of spermatogonial differentiation at the end of the 10-week GnRH-agonist treatment observed when the block was induced by DBCP appeared to be slightly greater than with radiation but slightly less than with procarbazine treatment. However, spermatogonial differentiation progressively increased in the case of irradiated testes following the cessation of GnRH-agonist treatment, but it declined in the DBCP- or procarbazine-exposed testes. It should be noted, however, that in other experiments the restoration of spermatogonial differentiation after irradiation by GnRH analogs also eventually declined (Shuttlesworth and Meistrich, unpublished).

Unlike our previous studies with radiation (Meistrich et al., 2001) or procarbazine (Meistrich et al., 1999), we were unable to prove that GnRH-analog treatment significantly enhanced fertility after DBCP exposure, despite the similar stimulation of spermatogenic recovery. We believe that this was a consequence of the lesser ability of GnRH-agonist treatment to produce sustained spermatogonial differentiation after cessation of the hormone treatment and the greater intraexperiment and interanimal variation in the gonadal response to DBCP than to radiation or procarbazine. Although it is possible that

**TABLE 1**

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>Duration of hormone treatment (weeks)</th>
<th>Time after DBCP treatment (weeks)</th>
<th>Sertoli cells per cross section</th>
<th>A spermatogonia per 100 Sertoli cells</th>
<th>BrdU labeling index (%)</th>
<th>Mitotic index (%)</th>
<th>Apoptotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>n.a.</td>
<td>11.6</td>
<td>13.5 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>44.9 ± 2.4</td>
<td>7.2 ± 1.6</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>GnRH agonist</td>
<td>5.6</td>
<td>11.6</td>
<td>12.1 ± 0.5*</td>
<td>1.3 ± 0.1</td>
<td>50.7 ± 4.8</td>
<td>5.9 ± 1.2</td>
<td>4.0 ± 1.5</td>
</tr>
<tr>
<td>None</td>
<td>n.a.</td>
<td>16</td>
<td>12.4 ± 0.8</td>
<td>1.4 ± 0.2</td>
<td>52.4 ± 5.4</td>
<td>5.6 ± 0.9</td>
<td>5.2 ± 1.2</td>
</tr>
<tr>
<td>GnRH agonist</td>
<td>10</td>
<td>16</td>
<td>10.0 ± 0.3*</td>
<td>1.9 ± 0.1</td>
<td>49.6 ± 4.2</td>
<td>5.9 ± 1.2</td>
<td>3.7 ± 0.5</td>
</tr>
</tbody>
</table>

*Values presented as mean ± SEM for atrophic tubules without additional damage.

*GnRH-agonist treatment with leuprolide was started 6 weeks after DBCP and GnRH agonist levels were maintained either for 5.6 weeks or for 10 weeks. Rats were killed at 11.6 or 16 weeks after DBCP treatment, respectively.

*Significantly different from corresponding group not treated with GnRH agonist, P < 0.05 (t-test).
the additional tubular or possible epididymal damage (Kluwe et al., 1983) produced by DBCP might also be factors and that testicular sperm production may not be reflective of epididymal sperm count, they are not major factors because the late spermatid count separating the fertile from the infertile group of rats (about $2 \times 10^7$) is similar to that observed after radiation alone ($1 \times 10^7$) or radiation followed by GnRH-agonist treatment ($4 \times 10^7$) (Meistrich et al., 2001). In any case, the results show that late spermatid count can be significantly enhanced in DBCP-treated rats by a GnRH agonist (Fig. 4b) and that fertility is related to late spermatid count (Fig. 5).

One could expect that the testicular damage observed after DBCP would be similar to that observed after procarbazine or busulfan, since the proposed mechanisms of DBCP action also include alkylation and formation of adducts with DNA bases (Humphreys et al., 1991; Pearson et al., 1990). However, the other agents and aging have different mechanisms of action, and some, like hexanedione, target the Sertoli cells whereas others, like radiation, primarily target the germ cells (Lee et al., 1999). The common factor in the ability of GnRH analogs to stimulate the recovery of spermatogenesis after a wide variety of toxicants appears to be that in all cases the inhibition of spermatogenesis is at the level of the differentiation of type A spermatogonia.

The reversibility of the block in spermatogonial differentiation by GnRH-analog treatment after exposure to other toxicants led to the suggestion that it was largely the elevated intratesticular levels of testosterone, but possibly also FSH, acting on the testis that caused this inhibition (Meistrich et al., 1999; Meistrich and Shetty, 2003; Shetty et al., 2000, 2002). The present results, showing the prevention and reversal of DBCP-induced testicular damage, support that hypothesis, since both the ITT and FSH levels were high in the DBCP-treated rats and were reduced by the GnRH-agonist treatment. But the reduction in ITT was greater when a 10-week GnRH-agonist treatment was given immediately after DBCP than when it was started 6 weeks later (Fig. 2d). The differences in ITT reduction could be responsible for the greater stimulation of the TDI (to 94%) with immediate treatment (Fig. 3) than with delayed treatment (52%) (Fig. 6). A similar relationship was noted after irradiation: The suppression of ITT and the stimulation of recovery of spermatogenesis were both greater when the GnRH agonist was given immediately as opposed to 20 weeks after irradiation (Meistrich et al., 1999). Preliminary data (G. Shetty, unpublished observations) also indicated that both the abilities to suppress ITT and to restore spermatogenesis in mutant jsd (juvenile spermatogonial depletion) mice with GnRH analogs were reduced with mouse age and further decline in spermatogenesis. Thus, the decreased effectiveness of delayed hormonal treatment may be due to its decreased ability to suppress ITT, rather than to a decrease in the intrinsic responsiveness of the cells. More effective suppression of testosterone levels or action might restore the effectiveness of stimulation of recovery.

In these instances of blocked spermatogonial differentiation, the spermatogonia actively proliferated but died by apoptosis instead of differentiating. We previously reported that GnRH-agonist treatment initiated 15 weeks after irradiation altered spermatogonial kinetics (Shuttlesworth et al., 2000). The number of type A spermatogonia was increased within 1 week, and the increase was maintained. There was also an increase in the labeling index of the type A spermatogonia. Although there may have been a slight increase in the numbers of type A spermatogonia and a decrease in their apoptotic indices in the atrophic tubules with GnRH-agonist treatment after DBCP-induced testicular atrophy, these changes were not statistically significant, and there was no increase in labeling index of the spermatogonia (Table 1). Whereas GnRH-agonist–treated irradiated rats also showed a transient decline in mitotic and apoptotic indices for the first week or two after initiation of GnRH-analog treatment, these levels returned to those seen in the irradiated-only rats by about 5 weeks (Shuttlesworth et al., 2000). Two reasons that changes in spermatogonial kinetics were not observed in the present study could be that we first counted type A spermatogonia after 5.6 weeks of GnRH-agonist treatment, perhaps missing evidence of transient

---

**TABLE 2**

Comparison of Stimulation of Spermatogonial Differentiation with GnRH-Agonist Treatment after Exposure to DBCP, Radiation, or Procarbazine

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>Time after toxicant exposure (weeks)</th>
<th>DBCP (350 mg/kg, present study)</th>
<th>Radiation (3.5 Gy, Meistrich et al., 1999)</th>
<th>Radiation (6 Gy, Shetty et al., 2000)</th>
<th>Procarbazine (250 mg/kg, Meistrich et al., 1999)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
<td>36 ± 11</td>
<td>54 ± 10</td>
<td>1 ± 0</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>GnRH agonist</td>
<td>10</td>
<td>36 ± 1</td>
<td>54 ± 10</td>
<td>1 ± 0</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>None</td>
<td>20</td>
<td>30 ± 5</td>
<td>93 ± 2</td>
<td>58 ± 5</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>GnRH agonist</td>
<td>20</td>
<td>100 ± 0</td>
<td>91 ± 4</td>
<td>91 ± 4</td>
<td>89 ± 7</td>
</tr>
</tbody>
</table>

*LBNF: rats were treated, starting immediately after exposure to the toxicant, with the GnRH agonist leuprolide, the levels of which were maintained for about 10 weeks by injections every 23–24 days. The dose of leuprolide was 1.83 mg per injection in all studies except in the one employing 3.5 Gy of irradiation, in which it was 1.25 mg.
changes, or that the changes occurred only in the subset of tubules in which repopulation was induced, but other explanations are possible too. Further studies are required to determine whether a decrease in apoptosis of type A spermatogonia at a particular stage of development is indeed responsible for the further development of spermatogenesis in a subset of tubules.

The mechanism by which testosterone and FSH inhibit spermatogonial differentiation in toxicant-treated rats and, consequently, how suppression of these hormones stimulates the recovery of spermatogenesis is still not known. Several important questions must be addressed in order to elucidate the mechanism as has been reviewed recently (Meistrich and Shetty, 2003). It must be determined whether the block to spermatogonial development is a result of a toxic effect on the spermatogonia themselves or on the Sertoli or other somatic cells. It also must be determined whether the failure of spermatogonial differentiation is a result of the loss of an essential growth or differentiation factor pathway or the induction of an apoptotic pathway that results in spermatogonial death before they are able to differentiate. In addition, the role of testicular edema, which occurs after both irradiation and DBCP treatment, needs to be addressed. Once the cellular and physiological targets for the testosterone-induced block in spermatogonial development is elucidated, it will be useful to screen for differentially expressed genes that are related to the block to spermatogonial development. We are currently addressing these questions using the irradiated rat model (Meistrich and Kangasniemi, 1997) because of the greater reproducibility of the response and the absence of systemic toxicity.

The present study has emphasized that the same principles apply to a wider range of toxicants, including important occupational and environmental agents. Although DBCP has been banned, its environmental concentrations are decreasing, and many of the exposed individuals are aging beyond their reproductive years, similar compounds are still widely used. For example 1- and 2-bromopropanes are widely used solvents that cause reproductive toxicity in both rats and humans (Ichihara et al., 2000; Kim et al., 1996). Like DBCP, 2-bromopropane is selectively toxic to spermatogonia and produces DNA damage (Omura et al., 1999; Wu et al., 2002).

These results indicate that there may be some possibility for the restoration of spermatogenesis in men rendered sterile by DBCP and other reproductive toxicants. The ability to reverse the decline in sperm counts depends on the existence of surviving stem spermatogonia that fail to complete differentiation. Although histological studies on testes of men exposed to high doses of DBCP often indicate the absence of any germ cells, some azoospermic individuals have occasional spermatogonia and even more advanced germ cells (Potashnik et al., 1978; Potashnik and Yanai-Inbar, 1987). The occasional spontaneous reversal of DBCP-induced azoospermia or oligospermia further indicates that there are stem cells that are capable of additional activation and differentiation into spermatogonia. Further investigation is needed to determine whether the principles demonstrated here for the restoration of spermatogenesis in rats by transient suppression of gonadotropins and testosterone will apply to humans, since the potential applications are great and the toxicity of the treatment is low.

ACKNOWLEDGMENTS

This work was supported by Research Grant ES-08075 (M.L.M.), Core Grant CA-16672, Training Grant CA-77050 (K.L.P.) from NIH, and a fellowship from the Lalor Foundation (G.S.). We thank Dr. Jerrold Heindel for valuable discussions and encouragement in the initiation of these studies, Dr. Pirjo Pakarinen and Ms. Tarja Laiho for gonadotropin measurements, Marley Herrin and Takeshi Yamada of TAP Pharmaceuticals for providing the Lupron, Kuriakose Abraham for preparation of the histological samples, and Walter Pagel for editorial assistance.

REFERENCES


