EFFECTS OF THE FUNGICIDE METHYL-BENZIMIDAZOL-2-YL CARBAMATE (MBC) ON MOUSE GERM CELLS AS DETERMINED BY FLOW CYTOMETRY

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Dual-parameter (DNA, RNA) flow cytometry (FCM) measurements were made on testicular and epididymal sperm cells isolated from mice exposed by oral gavage to 0, 250, 500, or 1000 mg/kg × 5 d of the fungicide methylbenzimidazol-2-yl carbamate (MBC), which is known to bind with tubulin subunits and inhibit polymerization and microtubule formation. Effects of exposure to MBC were measured at 7, 24, and 39 d posttreatment. MBC had no effect on body weights, but testis weights and sperm parameters were altered, with few exceptions, only at the highest exposure level. Testis weights were reduced by about 25% at 7 and 24 d after exposure; recovery was observed by 39 d after treatment. FCM measurements of testicular cells showed relative percentages of certain testicular populations (round, elongating, and elongated spermatids) were different from the control pattern 7 and 24 d after treatment.

The mean percent of cauda epididymal sperm head morphology abnormalities and the susceptibility of the nuclear DNA to denaturation were both elevated at 7, 24, and 39 d after exposure to 1000 mg/kg. The level of denaturation was determined by FCM measurements of the metachromatic shift in acridine orange (AO) stained sperm nuclei from green (native DNA) to red (single-stranded DNA) fluorescence and quantitated by the expression α_t[red/(red + green] fluorescence.

These data demonstrate that spermatogenesis is sensitive to high-dose MBC exposure resulting in an altered ratio of testicular cell types present, abnormal sperm head morphology, and an altered sperm chromatin structure.

INTRODUCTION

The antimitotic systemic fungicide methyl-benzimidazol-2-yl carbamate (MBC) is used for disease control in a wide range of crops. MBC is also formed in plant cells as a conversion product of other systemic

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fungicides (i.e., by cyclization of thiophanate-methyl and hydrolysis of benomyl) and is thought to be responsible for their fungicidal properties (Bent, 1979; Gardiner et al., 1974).

MBC binds to fungal tubulin subunits and, to a lesser extent, with plant and mammalian tubulin, preventing polymerization and microtubule formation (Davidse and Flach, 1977; Friedman and Platzer, 1978; Ireland et al., 1979). Mammalian tissue culture cells treated with MBC or serum from rats dosed orally with MBC exhibited cytotoxic effects including mitotic arrest, mitotic delay, and a low incidence of chromosome damage (Styles and Garner, 1974).

Oral administration of relatively low doses of MBC (Delatour and Richard, 1976) and benomyl, its parent compound, during gestation caused fetotoxicity and teratogenesis in rats (Schentenberg and Torchinskiy, 1972; Kavlock et al., 1982) and mice (Kavlock et al., 1982). Oral treatment with 31.2 mg benomyl/kg · d during gestation and lactation in rats also induced permanent reductions in testes and accessory sex gland weight in adult male offspring (Kavlock et al., 1982).

Oral dosing of benomyl (0–1000 mg/kg · d) to adult male rats has been reported to have adverse effects on the testes and other reproductive organs, including changes in testicular biochemistry, degeneration of testicular tissue (hypospermatocytogenesis and hypospermatogenesis), decreased testicular and epididymal weights, reduced number of testicular spermatozoa, and depressed sperm concentrations in the epididymis and vas deferens (Torchinskiy et al., 1976; Carter and Laskey, 1982; Carter et al., 1984). Carter et al. reported that Sprague-Dawley male rats exhibited an "age-related" difference in sensitivity to oral doses of benomyl (0–1000 mg/kg · d) with only pubertal and postpubertal animals showing significant treatment effects. Barnes et al. (1983) reported that 70 d after completion of benomyl administration (0–203 ppm in feed/d × 70 d), i.e., 140 d after initiation of benomyl administration, treatment-induced alterations in testicular function of rats were reversed with male fertility, ejaculate sperm content, and testicular weight returning to control levels. Carter and Laskey (1982) suggested that changes in testicular function due to benomyl treatment may result from the effect of MBC on highly tubulin-dependent events of spermatogenesis after conversion of benomyl to MBC.

Recently, dual-parameter flow cytometry (FCM) protocols utilizing the metachromatic fluorochrome acridine orange (AO) (Darzynkiewicz et al., 1976) were used to characterize cells in immature (Janca et al., 1986) and adult mammalian testis (Evenson and Melamed, 1983; Evenson et al., 1986a) and epididymal sperm chromatin structure (Evenson et al., 1980, 1985, 1986b). The latter studies showed that strong alkylating agents altered spermatogenesis, including sperm chromatin structure, so that the DNA became more susceptible to acid or heat induced denaturation in situ. The study reported here was done primarily to

determine whether MBC, a nonalkylating agent and inhibitor of tubulin polymerization, would affect sperm chromatin structure in a manner that also would alter the resistance of DNA to denaturation in situ.

METHODS

Seven-week-old C57BL/6J × C3H/HeJ F_1 male mice were obtained from Jackson Laboratories (Bar Harbor, Me.) and allowed to acclimate 12 wk at our facilities. Animals were housed in plastic cages with stainless steel wire tops, maintained on an ad libitum diet of Purina Rodent Chow (Ralston Purina Co., St. Louis, Mo.) and deionized water, and kept at constant room temperature (21 ± 2°C) with a lighting schedule of 0700–1900 h.

Individual mice were weighed and randomly assigned to 4 dosage levels of technical grade (98.1%) methyl-2-benzimidazolecarbamate (MBC; E. I. DuPont De Nemours and Co., Wilmington, Del.). Mice were dosed, via oral gavage, daily for 5 consecutive days with 0.2 ml corn oil (Sigma Chemical Co., St. Louis, Mo.) alone (control) or containing freshly prepared suspensions of MBC calculated to deliver 0, 250, 500, and 1000 mg MBC/kg body weight (mg/kg). Computed values were from mean daily weight (averaging 31 g for each of the 5 d) of all treated animals. Oral gavage was performed using an 18 gauge 1½-in. curved feeding needle with 1¼-mm ball tip (Popper and Sons, Inc., New Hyde Park, N.Y.).

Four animals per dose level were killed 7, 24, and 39 d posttreatment. Body and testis weights were measured immediately after killing by cervical dislocation. Testes and cauda epididymides (cauda) were excised and immediately placed in cold (4°C) buffer solutions specified below. Testes and cauda tissues were minced into cellular suspensions with curved surgical scissors, transferred to 12 × 75 mm conical polystyrene culture tubes (Fisher Scientific Co., Pittsburgh, Pa.), and after settling of cell clumps were filtered through Nitex nylon filters (Tetko, Inc., Elmsford, N.Y.) attached to tuberculin syringes (Becton, Dickinson & Co., Rutherford, N.J.). Cell preparations were kept at 4°C during all subsequent procedures.

Testicular Cell Suspensions

One testis from each animal was minced with scissors in 2 ml Hanks balanced salt solution (HBSS; Gibco Laboratories, Grand Island, N.Y.). The resulting cell suspensions were filtered through 53-μm nylon filters.

Cauda Sperm Cells

Cauda epididymides from each animal were minced in 2.0 ml TNE buffer (0.15 *M* NaCl, 0.01 *M* Tris-HCl, 1 m*M* EDTA-Na^{2+}, pH 7.4) and

liberated cells filtered through 153-μm nylon filters. Two aliquots per filtered sample were mixed 1:1 with 1% eosin Y (in water), stained for 30 min (22°C), and then smeared onto glass microscope slides. After air-drying, slides were dipped into methanol to remove excess stain and coverslipped using Permount (Fisher Scientific Co., Pittsburgh, Pa.). Then 350 intact sperm cells from each of 4 mice per dose per time group were scored by light microscopy for sperm head abnormalities according to the criteria of Wyrobek and Bruce (1975).

Acridine Orange Staining of Germ Cells

Testis Cells. Approximately 2–4 × 10^5 cells in 200 μl HBSS were admixed with 400 μl solution containing 0.08 *N* HCl, 0.15 *M* NaCl, and 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) according to the method of Darzynkiewicz et al. (1976). Triton X-100 partially dissolves cell membranes, facilitating dye penetration, while the low pH dissociates histones from histone-containing testicular cells (Evenson et al., 1980). Thirty seconds later, 1.2 ml staining solution containing 6 μg/ml acridine orange (AO; chromatographically purified, Polysciences, Warrington, Pa.) in AO buffer [0.2 *M* Na_2HPO_4, 0.1 *M* citric acid buffer (pH 6.0), 1 m*M* EDTA, 0.15 *M* NaCl] was added to the sample.

When excited by blue laser light (488 nm), AO molecules intercalated into double-stranded (ds) nucleic acids emit green fluorescence (F_{530}) (Lerman, 1963); red fluorescence ($F_{>630}$) is emitted from AO associated with single-stranded (ss) nucleic acids (Bradley and Wolf, 1959). FCM measurements of red and green emitted from stained testicular cells (spermatogonia through spermatids) are related to RNA and DNA stainability, respectively (Evenson and Melamed, 1983; Evenson et al., 1986b).

Caudal Sperm. Caudal sperm were stained by the above procedure, which has been termed the sperm chromatin structure assay (SCSA) (Evenson et al., 1985; Evenson, 1986). The 30-s, low-pH treatment partially denatures sperm DNA with abnormal chromatin structure. Red and green fluorescence reflect amounts of AO-accessible single- and double-stranded DNA, respectively (Evenson et al., 1985, 1986b). Apparently, the protamine-complexed DNA is susceptible to acid-induced denaturation, while that complexed with histones in testicular germ cells or other somatic cells is resistant under the staining conditions used (Evenson et al., 1985; Darzynkiewicz et al., 1976). Sperm in suspension were also sonicated as previously described (Evenson et al., 1986b) to remove potential RNA-containing cytoplasmic droplets that could interefere with the red fluorescence related to single-stranded DNA.

Flow Cytometry (FCM)

Immediately after AO staining, approximately 200 cells/s were passed through the quartz flow channel of a Cytofluorograf II (Ortho Diagnostic Systems, Inc., Westwood, Mass.) equipped with ultrasense optics and a Lexel 100-mW argon ion laser operating at 35 mW (488 nm). Total fluorescence from each cell was collected by a lens situated at right angles to the sample flow and laser beam. By use of dichroic mirrors and filters, the fluorescent signals were separated into green (515–530 nm) and red (>600 nm) components. A slightly modified preamplifier circuit board (Ortho Diagnostic Systems, Inc.) was used to reduce background fluorescence of AO in the sample stream.

The stained samples are stable over a period of time (≥30 min); however, for uniformity, recorded measurements of each sample were begun 3 min after staining. The amplified and digitized signals were viewed in live display on the cathode-ray tube (CRT) screen of the interfaced 2150 Data Handler (Ortho Diagnostic Systems, Inc.) and also stored on computer disks in list mode. Hard copies of the data were made with a Tektronics 4612 copier (Tektronics, Inc., Beaverton, Ore.). Data were based on 5×10^3 cells/sample.

Data Analysis

Correlations of experimental variables were computed using the Statistical Analysis System (SAS Institute, Inc., Cary, N.C.) procedures on an IBM mainframe computer. A significance level of $p < 0.05$ or better is used throughout.

RESULTS

Body and testis weights were determined 7, 24, and 39 d posttreatment with 0, 250, 500, and 1000 mg MBC/kg. Data from the treated groups did not significantly differ from the controls, except the ratios of testis weights from the mice exposed to the highest dose (1000 mg/kg) to the control testis weights were 0.72, 0.76, and 0.88 at 7, 24, and 39 d posttreatment. Table 1 shows the correlations of MBC dosage with testicular variables and body weights.

The mean relative percentages ($\bar{x}$%) of testicular cell populations [haploid (1*n* cell types verified by light microscopy identification of FCM-sorted cell types), diploid (2*n*), and tetraploid (4*n*)] were determined as previously described (Evenson et al., 1985). All significant changes in mean relative percentages of these populations occurred at 7 and 24 d posttreatment at 1000 mg/kg. An exception was in the 500 mg/kg group, 24 d posttreatment, where the $\bar{x}$% of round spermatids was reduced >1 SD from the control mean (±1 SD). Note in Fig. 1 that the total 1n population decreased by d 24; however, within that popu-

TABLE 1. Correlations of MBC Dosage[a] with Testicular Variables and Body Weights

Parameter	Days posttreatment		
	7	24	39
$\bar{x}$ % Round spermatids	0.85[e]	0.83[e]	0.39
$\bar{x}$ % Elongating spermatids	−0.90[e]	−0.16	−0.47
$\bar{x}$ % Elongated spermatids	—	−0.89[e]	0.11
$\bar{x}$ % 1*n* Cell types	−0.62[c]	−0.75[d]	−0.51
$\bar{x}$ % 2*n* Cell types	0.74[d]	0.67[c]	0.29
$\bar{x}$ % 4*n* Cell types	−0.43	0.76[d]	0.48
$\bar{x}$ Testis weight	−0.82[d]	−0.71[c]	−0.57
$\bar{x}$ Body weight	−0.15	−0.13	−0.56

[a] Dosages used were 0, 250, 500, and 1000 mg/kg.
[b] $n = 12$ for each time point.
[c] Significant at $p < 0.05$.
[d] Significant at $p < 0.01$.
[e] Significant at $p < 0.001$.

lation, the relative percentage of round spermatids was higher and elongated spermatids lower than the control population. By d 39 the ratios of the various cell types were returning to normal.

Epididymal Sperm

Mean percent cauda sperm head morphology abnormalities (% ABN; Fig. 2) were significantly affected by MBC 7 and 24 d posttreatment (1000 mg/kg) and 39 d posttreatment (500 and 1000 mg/kg). Of interest, the % ABN was the same value at 7 and 24 d (8.8 ± 6, 8.4 ± 2.2, respectively) and reduced (6.6 ± 2.3) at 39 d posttreatment.

The effect of MBC on mouse sperm chromatin structure, as defined by changes in the susceptibility of DNA to acid-induced denaturation in situ, was determined by FCM measurements on AO-stained sperm. The change was measured by the shift from green fluorescence (AO intercalated into native DNA) to red fluorescence (AO associated with single stranded DNA) and quantitated by the expression α_t[red/(red + green)] fluorescence. The SD of α_t of acridine orange-stained cauda sperm responded to 1000 mg/kg MBC treatment (Fig. 2). At 7 d posttreatment, the SD of α_t was 6 times greater than control mean values and was positively correlated with % ABN; $r = 0.77$ ($p < 0.01$). At 39 d posttreatment (1000 mg/kg), SD α_t was outside control mean values (±2 SD) and was positively correlated with % ABN.

DISCUSSION

MBC was shown here to have a significant effect on testicular cell population kinetics and sperm chromatin structure only at high doses

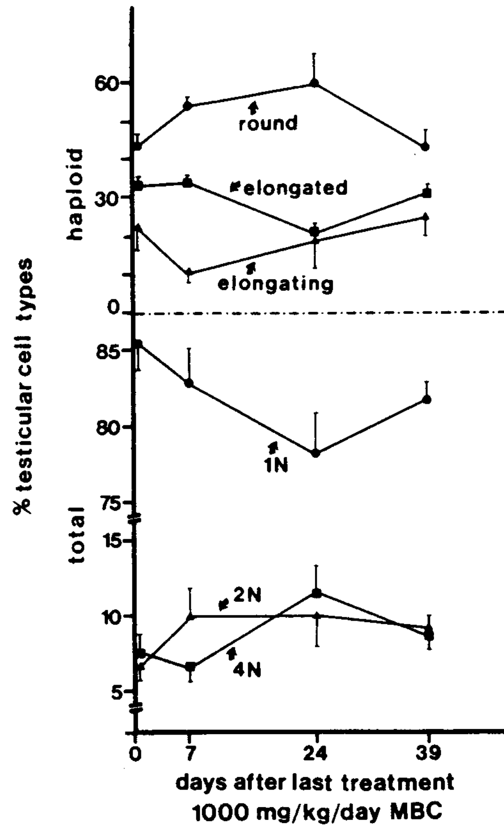

FIGURE 1. Plot of changes in $\bar{x}$ ± SD % of testicular cell types 7, 24, and 39 d posttreatment with 1000 mg MBC/kg · d (×5). Mean values obtained from 9 control animals at 7, 24, and 39 d post-treatment were used for 0-dose (control) values.

(1000 mg/kg), with moderate effects at 500 mg/kg depending on the recovery period following treatment. Since 93% of [2-^{14}C]MBC is eliminated from rodents within 24 h after oral exposure (Gardiner et al., 1974), testicular cells in animals given oral doses would be exposed to MBC primarily during the treatment period. The data suggest that there are several periods during spermatogenesis when cells are sensitive to high doses of MBC. The 50% reduction in mean percent elongating spermatids at 7 d posttreatment indicates one susceptible time period must encompass the midpachytene through early round spermatid stages, which the majority of elongating spermatids passed during exposure (Fig. 3). These stages include diakinesis through meiosis I and II during d 2–5 of treatment. Since meiosis I and II each require the formation of spindle apparatus and other tublin-requiring structures, these cell stages are likely to be the most sensitive as the data indicate.

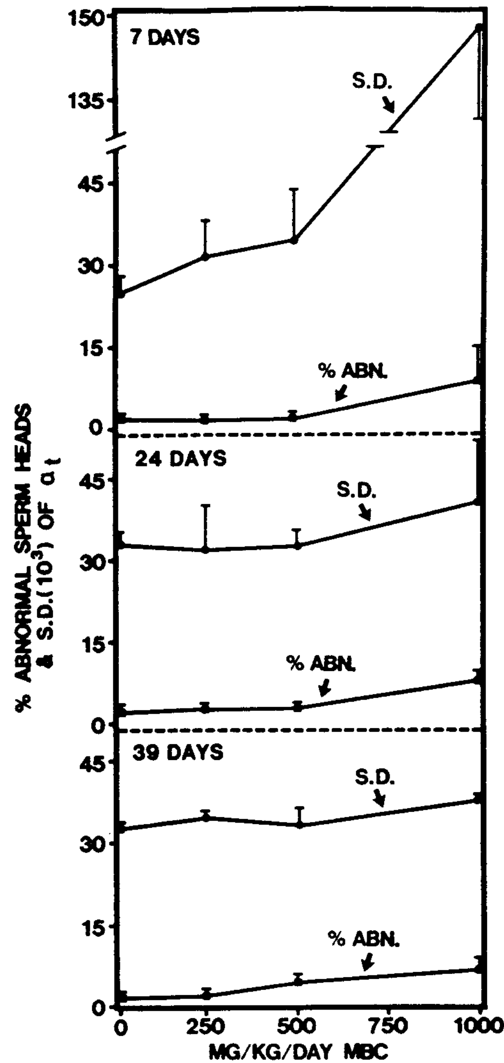

FIGURE 2. Plot of % ABN among cauda sperm and SD ($\times 10^3$) of α_t 7, 24, and 39 d posttreatment with corn oil–MBC suspensions [0, 250, 500, and 1000 mg MBC/kg · d ($\times 5$)]. Each value is expressed as $\bar{x} \pm$ SD (n = 4). The multiplier 10^3 was used for SD α_t to facilitate overlaying plots on the same scale as % ABN.

Since the mean percent of round spermatids at 7 d posttreatment did not decrease, treated cell stages that gave rise to these round spermatids, i.e., late leptotene through middiplotene spermatocytes, can be excluded as being sensitive (Fig. 3). Likewise, the mean percent elongated spermatids showed little or no decrease at 7 d, suggesting that round and more advanced spermatids were not sensitive to MBC, at least relative to production of that succeeding cell type.

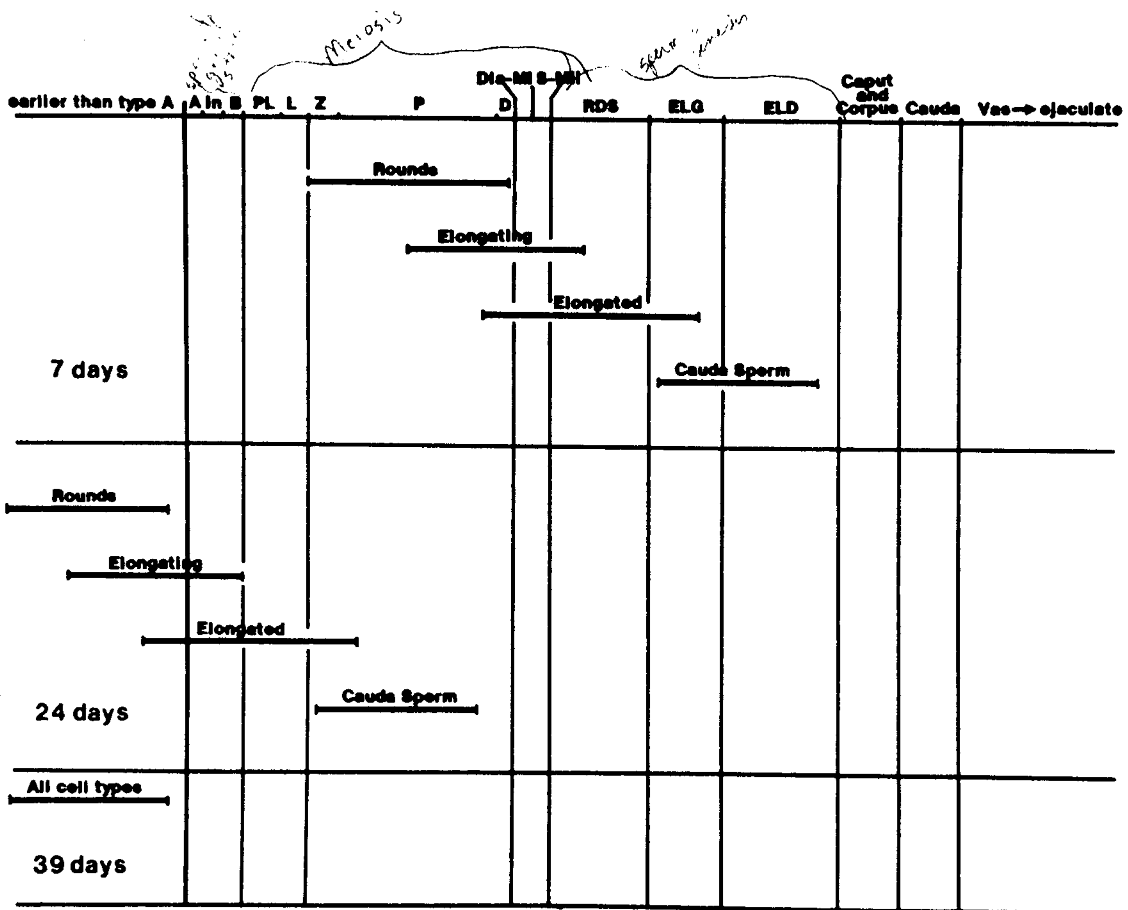

FIGURE 3. Diagram representing the spermatogonial stages during the 5-d MBC treatment period, which gave rise to round, elongating, and elongated spermatids and cauda spermatozoa 7, 24, and 39 d posttreatment. Bars show stages during which cells were treated. Spermatogonial stages: type A spermatogonia (A), intermediate spermatogonia (In), type B spermatogonia (B). Meiotic stages: preleptotone (PL), leptotene (L), zygotene (Z), pachytene (P), diplotene (D), diakinesis-reductional division (Dia-MI), secondary spermatocyte–equational division (S-MII). Spermatid stages: round spermatid (RDS), elongating spermatid (ELG), elongated spermatid (ELD). Duration of spermatogenic stages is based on Oakberg (1956a,b); timing of sperm movement through epididymis is from Sega (1974).

All round and elongating spermatids measured at 24 and 39 d posttreatment would have been type B spermatogonia or earlier cells when treated (Fig. 3). Since the mean percentages of round and elongating spermatids were not significantly reduced at these times, MBC does not appear to have a detectable cytotoxic or inhibitory effect on early spermatogonial cells. At 24 d posttreatment, a significant decrease in mean 1*n* cells is correlated with a significant decrease in elongated spermatids, which were exposed as type A spermatogonia through early pachytene spermatocytes. Spermatogenic stages from late type B spermatogonia to early zygotene spermatocytes were unique to treated cells which were elongated spermatids 24 d posttreatment. Since zygotene spermatocytes were apparently not MBC-sensitive after 7 d posttreatment, only the period including late type B spermatogonia through leptotene spermatocytes appears to contain cells sensitive to MBC.

By 39 d posttreatment the distribution of testicular cell types was normal and cells treated as mitotically proliferating type A spermatogonia or earlier cell stages populated the seminiferous tubules.

Spermatogonia passing through the latter part of spermatogonial proliferation to early meiosis undergo substantial changes in cell size and chromatin structure (Bellve et al., 1977). The preleptotene–leptotene spermatocyte phases are characterized by events that are unique to meiosis, resulting in the production of haploid gametes. These events include the last DNA synthesis prior to meiosis, initiation of synaptonemal complex assembly, and concurrent synapsis of homologous chromosomes. Some of these events are dependent on tubulin polymerization, and changes in polymerization rate due to MBC binding could have adverse effects on chromosome segregation and condensation. Tubulin probably plays a role in sperm head shaping, and since the shape of the head is the same as demembranated nuclei, tubulin may also be involved in the ordering of gross chromatin structure. Previous studies have shown a dose response for strong alkylating agents on sperm head morphology (Wyrobek et al., 1975; Evenson et al., 1985, 1986b). The later studies (Evenson et al., 1985, 1986b) also showed a correlation of sperm head shape with altered chromatin structure as defined by the metachromatic shift of acridine orange staining. We are interested in determining the mechanism of this change in chromatin structure. We have ruled out lack of exchange of histones for protamines and the presence of RNA (Evenson et al., 1985). Since sperm obtained from mice exposed to alkylating agents could have had regions of depurinated DNA or DNA with single-strand breaks, these studies were intended to determine whether a nonalkylating agent would produce a similar effect.

Sperm cells sampled 7 d posttreatment were exposed to MBC as early elongating spermatids and elongated spermatids (Fig. 3). The cor-

relation between increased SD α_t values of whole sperm and % ABN 7 d posttreatment [$r = 0.89$ ($p < 0.001$)] strongly suggests that MBC-induced alterations in sperm chromatin structure were related to the increased % ABN. Maturational changes occurring during this period—e.g., nuclear elongation and gross shaping of nuclear chromatin—could be related to tubulin-requiring events, and MBC inhibition of these events may have led to alterations of chromatin structure and sperm head morphology that rendered the DNA less resistant to acid-induced denaturation. Repeat measurements on sperm heads separated from tails by sonication and purified through sucrose gradients showed the same α_t distribution. These results are consistent with earlier studies (Janca et al., 1986; Evenson et al., 1985, 1986b) and provide evidence that increased α_t values were not due to abnormal RNA retention during sperm maturation.

Germ cells that gave rise to cauda sperm 24 and 39 d posttreatment were treated during meiotic prophase (zygotene–pachytene) and type A spermatogonia (or earlier) stages, respectively (Fig. 3). MBC treatment during these spermatogenic stages did not appear to affect subsequent formation of spermatid types 7, 24, and 39 d posttreatment (Figs. 1 and 3). However, treatment at these stages did result in a significant increase in % ABN among cauda sperm 24 and 39 d posttreatment. Increases in SD α_t 39 d posttreatment (1000 mg/kg) accompanied the increase in % ABN. Cells treated during these stages may not have suffered immediate cytotoxicity or developmental inhibition, but could have sustained permanent genetic damage manifested several cell generations later as abnormal sperm head morphology and altered chromatin structure as seen in other studies (Evenson et al., 1986b).

These studies have shown that MBC has an effect on testicular cell kinetics, sperm head morphology, and sperm chromatin structure. Although MBC reduced the resistance of nuclear DNA to acid-induced denaturation, similar to that observed for alkylating agents, it is not known whether the mechanism of change is the same.

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