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Toxicity of Thiotepa on Mouse Spermatogenesis as Determined by Dual-Parameter Flow Cytometry

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Toxicity of Thiotepa on Mouse Spermatogenesis as Determined by Dual-Parameter Flow Cytometry. EVENSON, D. P., BAER, R. K., JOST, L. K., AND GESCH, R. W. (1986). *Toxicol. Appl. Pharmacol.* **82,** 151–163. Multiparameter flow cytometry (FCM) measurements were made on acridine orange (AO)-stained mouse testicular cells and epididymal sperm cells to determine the effects of varying dosages of thiotepa (0–5 mg/kg ip daily × 5 days) on spermatogenesis at 7, 28, and 67 days after the last exposure (ALE). FCM multiparameter measurements included DNA stainability vs RNA content, peak amplitude vs integrated area of DNA fluorescent signal, and double-stranded DNA vs single-stranded DNA. Thiotepa exhibited dramatic damaging effects on the kinetics and/or cell kill of seven testicular cell types measured by dual-parameter flow cytometry. At 7 days ALE, one 4N cell type, likely the pachytene spermatocyte, was absent from the testes, and another was reduced by about 70%. By 28 days ALE, most of the germ cells were absent from the seminiferous tubules, and by 67 days ALE the testes were undergoing recovery of spermatogenesis with only half of the seminiferous tubules repopulated after treatment with 5.0 mg/kg. The dual parameters of DNA stainability vs RNA content provided better resolution of testicular cell types into distinct populations than the peak vs area processing of the green fluorescent signal of AO-stained cells. Dosage of thiotepa was significantly related to percentage of sperm head morphological abnormalities assayed by light microscopy. Utilizing the metachromatic properties of acridine orange, FCM measurements of the amount of single-stranded DNA induced within acid-stressed whole sperm or heat-stressed nuclei detected alterations of chromatin structure at the same minimal effective dose required to increase abnormal sperm head morphology. Epididymal sperm isolated from mice exposed to some concentrations of thiotepa had an increased percentage of free heads and tails. DNA in free heads denatured *in situ* to a greater extent than DNA in intact sperm.

Spermatogenesis represents one of the most rapidly proliferating and highly complex cell differentiating systems in mammals. Differentiation is unique with regard to haploid genome reduction, exchange of somatic-like nuclear histones for sperm-specific protamines, and species-specific sperm morphology. Sperm morphology is thought to be controlled by genetic mechanisms because various strains of mice have abnormal sperm head morphologies ranging from 1.2 to 70% (Wyrobek *et al.*, 1976). Previous studies (Wyrobek and Bruce, 1975; Topham, 1980) have described a sperm morphology assay that is sensitive in a dose–response manner to chemical exposure. Although mechanisms are not understood for these chemically induced alterations, the test has proven to be a reliable indicator for mutagenic chemicals, i.e., 100% of germ cell mutagens tested were positive for induction of sperm head morphology abnormalities (Wyrobek *et al.*, 1983).

Previous studies (Clausen *et al.*, 1977; Gledhill *et al.*, 1979; Evenson *et al.*, 1980a,b;

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Evenson and Melamed, 1983; Evenson, 1985) have shown the usefulness of flow cytometry (FCM) to study spermatogenic events. Compared to light microscope studies, FCM measurements: (a) are more rapid (1000 cells/sec); (b) are defined by machine specifications rather than the subjective human eye; (c) allow large numbers of cell measurements which provide high statistical significance; and (d) are unbiased since all cells entering the instrument are analyzed.

Whereas previous FCM studies have used single-parameter measurements of testicular cells, our recent studies have utilized dual-parameter (DNA- and RNA-staining) measurements. Also, the metachromatic property of acridine orange (AO) (green fluorescence, double-stranded nucleic acids; red fluorescence, single-stranded nucleic acids) has been used to measure structural integrity of nuclear chromatin, defined by the susceptibility of DNA to undergo partial denaturation *in situ* (Evenson *et al.*, 1980a, 1985). Because mature mammalian sperm cells contain virtually no RNA (Monesi, 1971), they have minimal red fluorescence with AO staining (Evenson *et al.*, 1980b) that is not reduced by RNAse digestion (Evenson *et al.*, 1985). Previous studies (Evenson *et al.*, 1985) have related a chemically induced increase of red fluorescence to an increased susceptibility to partial DNA denaturation *in situ*.

N,N',N''-triethylene thiophosphoramide (thiotepa) acts as a polyfunctional alkylating agent (Calabresi and Parks, 1980) and is an *in vivo* mutagen in *Drosophila* (Epstein and Shafner, 1968), rats (Jackson *et al.*, 1959), and mice (Machemer and Hess, 1971). Antispermatogenic activity has been demonstrated in mice (Parvinen and Parvinen, 1978; Meistrich *et al.*, 1982). This drug currently is used in treatment of breast and ovarian adenocarcinoma and for intracavitary malignant effusions from serosal surfaces (Physicians Desk Reference, 1983). Clinical use of thiotepa for treatment of primary malignant neoplasms has been implicated in the induction of acute leukemia (Allan, 1970; Greenspan and Tung, 1974; Perlman and Walker, 1973; Ruffner, 1974). Recently, Breau *et al.* (1984) have shown that thiotepa requires metabolic activation with an Aroclor 1254-induced rat liver homogenate preparation (S-9) for mutagenic activity in the histidine operon in strain TA100 of *Salmonella typhimurium*.

This study shows that thiotepa kills and/or severely disrupts proliferation and differentiation of various mouse testicular germ cell types as measured by FCM. Of greater significance, FCM measurements detected chemical-induced epididymal sperm chromatin structure alterations at the same minimal effective dose that caused an increase in sperm head morphology abnormalities.

METHODS

Mice. Seven-week-old male F_1 mice (C57BL/6J × C3H/HeJ), obtained from the Jackson Laboratory (Bar Harbor, Me.), were allowed to acclimate to animal holding facilities for 4 weeks prior to chemical exposure. Animals were housed in polycarbonate cages bedded with pine shavings and covered with wire mesh tops. They were allowed free access to rodent laboratory chow (Ralston Purina Co., St. Louis, Mo.) and deionized water. The animals were maintained on a constant 12-hr light:dark cycle with temperature held at 21 ± 2°C. Randomly selected mice (3 from each dosage group) were killed by cervical dislocation 7, 28, and 67 days after the last chemical exposure (ALE).

Chemical exposure. Nine mice were allotted randomly to each of four groups and injected: ip daily for 5 consecutive days with 0.5 ml Hank's balanced salt solution (HBSS; control, vehicle alone) or HBSS plus thiotepa (Lederle, Pearl River, N.Y.) providing daily exposures of 0, 0.5, 2.5, and 5.0 mg/kg body wt.

Testicular germ cells. Immediately after the animals were killed, the body weights were measured. Testes were removed and weighed; one was placed into a 60-mm petri dish containing HBSS at 4°C and the other fixed in 10% buffered Formalin and routinely processed for histologic examination. The testis in HBSS was minced with curved scissors and transferred to a 12 × 75-mm test tube. After several minutes to allow settling of testicular fragments, the supernatant fraction was filtered through 53 μm nylon mesh (Tetko, Inc., New York, N.Y.) placed between the end of a tuberculin syringe and its plastic protective cap (end cut off).

Epididymal Sperm

Fresh cells. The caudal portion of each pair of epididymi was excised, placed into 2 ml of TNE buffer (0.15 M NaCl,

0.01 M Tris, 1 mM EDTA, pH 7.4) in a 60-mm petri dish at 4°C, and minced with curved scissors. After aspirating and expelling gently several times through a 5-in Pasteur pipette, the sperm suspensions were filtered through 153 μm nylon mesh as above and kept in a test tube on crushed ice (4°C).

Isolated, fixed nuclei. Unused portions of epididymal sperm from mice 28 days ALE were pooled for each dosage group, diluted with TNE to a total vol of 12 ml in a 15-ml glass centrifuge tube, and centrifuged at 12,000*g* for 10 min at 4°C. The pellet was resuspended in 1.5 ml of TNE buffer and transferred to a Falcon No. 3033 plastic test tube which was sitting in a crushed ice-water slurry. The sample was sonicated (30 sec power, 30 sec cooling, 30 sec power) with a Bronwill Biosonik IV Sonicator (VWR Scientific Inc., Minneapolis, Minn.) equipped with a $\frac{3}{8}$-in. diameter probe, and operated at a setting of 50 on the low-power scale. The sonicate was admixed with 1.5 ml 60% sucrose w/w in 0.01 M Tris–HCl, 2 mM EDTA (pH 7.4), and 3 ml TNE, layered over 9 ml of the same sucrose solution in 16 ml Sepcor polycarbonate tubes with caps (Separation Science Corp., Stratford, Conn.), and centrifuged at 27,500*g* in a Sorvall HB4 rotor for 1 hr. The pellet was resuspended in 1.0 ml of a buffer (0.15 M NaCl, 0.02 M Tris–HCl, and 5 mM $MgCl_2$), pH 7.4, forcefully admixed with 9 ml of a 1:1 mixture of 70% ethanol and acetone (4°C), and stored at −20°C in a 15 × 150-mm glass tube with cap (Evenson *et al.*, 1980a).

Sperm morphology. Two drops of epididymal sperm suspension mixed with one drop of freshly filtered 1% eosin Y (H_2O) were incubated at room temperature for 30 min and then smeared onto a glass slide. After air drying, the slides were quickly dipped into methanol to remove excess stain, air-dried again, and coverslipped using Permount mounting medium (Fisher Scientific, Pittsburgh, Pa.).

Sperm head morphology was scored as normal or abnormal according to the criteria of Wyrobek and Bruce (1975). A minimum of 1000 nuclei from at least three animals per time/dose group were scored by use of a Nikon light microscope with a 100× oil immersion lens (total 1000×).

Cell Staining

Two-step acridine orange (TSAO) staining of fresh cells. Aliquots (0.20 ml) of testicular or epididymal sperm samples were admixed with 0.40 ml of 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.4. After 30 sec the cells were stained by adding 1.2 ml of a solution containing 6 μg chromatographically purified AO (Polysciences, Warrington, Pa.) per ml of AO buffer (0.1 M citric acid, 0.2 M $NaHPO_4$, 1 mM EDTA, and 0.15 M NaCl), pH 6.0 (Darzynkiewicz *et al.*, 1976; Evenson *et al.*, 1985). The stained samples were immediately placed into the flow cytometer sample chamber at 4°C and measured 3 min after staining.

The first step of the TSAO method utilizes Triton X-100 to punch holes in the cell membranes providing access for AO to enter the cells. The low pH of the solution dissociates histones from somatic cells (Darzynkiewicz *et al.*, 1976) and histone-containing testicular cells (Evenson and Melamed, 1983). This acid treatment increases the accessibility of DNA for AO staining in somatic cells and mature sperm cells by 2.5 and 1.3 times, respectively (Evenson *et al.*, 1980b). This low pH treatment apparently causes partial DNA denaturation in sperm with altered sperm chromatin structure (Evenson *et al.*, 1985). AO intercalates into native, double-stranded DNA and fluoresces green (F_{530}) after excitation by blue light. AO associated with single-stranded nucleic acid fluoresces red ($F \geqslant 600$) under these same conditions (Kapuscinski *et al.*, 1982). For somatic cells, or histone-containing germ cells, these staining conditions permit a stoichiometric determination of DNA and RNA content as verified by enzymatic digestion (Darzynkiewicz, 1979), staining relationships between G_1 and G_2 cells (Darzynkiewicz, 1979), and diploid and round spermatid testicular cells (Evenson and Melamed, 1983; Evenson *et al.*, 1986).

Thermal denaturation. Samples of previously isolated nuclei (from pooled epididymal sperm at each dosage level from 28 days ALE) were centrifuged for 10 min at 650*g* in the same tubes used for storage. The pellets of nuclei were resuspended in 5 ml heating buffer, pH 6.0, consisting of 0.002 M sodium cacodylate, 0.0001 M disodium EDTA, and 40% ethanol (v/v). The samples were allowed to equilibrate for 20 min on ice followed by centrifugation for 10 min at 16,000*g*. The pellet was resuspended in 1.0 ml of the above heating buffer and diluted if necessary to an approximate concentration of 1×10^6 nuclei/ml. FCM measurements were made on an AO-stained, unheated aliquot and a thermally denatured aliquot from each sample. For the unheated aliquot, 0.1 ml of the suspended nuclei was stained with 1.0 ml of staining solution (0.15 M NaCl, 0.005 M $MgCl_2$, 0.02 M Tris–HCl, pH 7.4, and 8 μg/ml AO) and measured by FCM after 3 min. For the heated sample, a 0.4-ml aliquot (increased volume to compensate for loss of nuclei by sticking to tube wall and volume by evaporation) of sample was transferred to a Falcon plastic test tube (No. 3033), heated in a boiling water bath for 5 min, and cooled for 15 sec in an ice-water slurry. The sample was then stained with 2.0 ml of the above staining solution, mixed gently, and measured by FCM 3 min later.

Fluorescence measurements. Stained cells were passed at a rate of about 200/sec through the quartz flow cell in a Cytofluorograf II flow cytometer (Ortho Diagnostics, Inc., Westwood, Mass.) equipped with ultrasense optics and a Lexel 100 mW argon ion laser operated at 35 mW and interfaced to a 2150 data handler, a Data General 6123 tape drive, and a Tektronix 4612 hard copy unit (Tektronix, Inc., Beaverton, Oreg.). A slightly modified preamplifier circuit board (Ortho Diagnostics, Inc.) was used to reduce background fluorescence of AO in the sample stream.

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. These were amplified, digitized, viewed on live display, and stored on computer disks in list mode. Hard copies were made of the live data in histogram and cytogram form to monitor progress of the ongoing experiment. Single-color fluorescence values were processed as area vs peak signals to further discriminate between different cell types and cell doublets. The data were based on measurements of 5000 cells/sample.

RESULTS

Body and testicular weights. Intraperitoneal exposure to thiotepa at concentrations of 0 to 5 mg/kg/day for 5 days had no significant effect on body or testicular weights 7 days ALE. The drug had no effect on body weights 28 and 67 days ALE, but dramatic losses (70 and 50%, respectively) were observed for testicular weights as shown in Fig. 1.

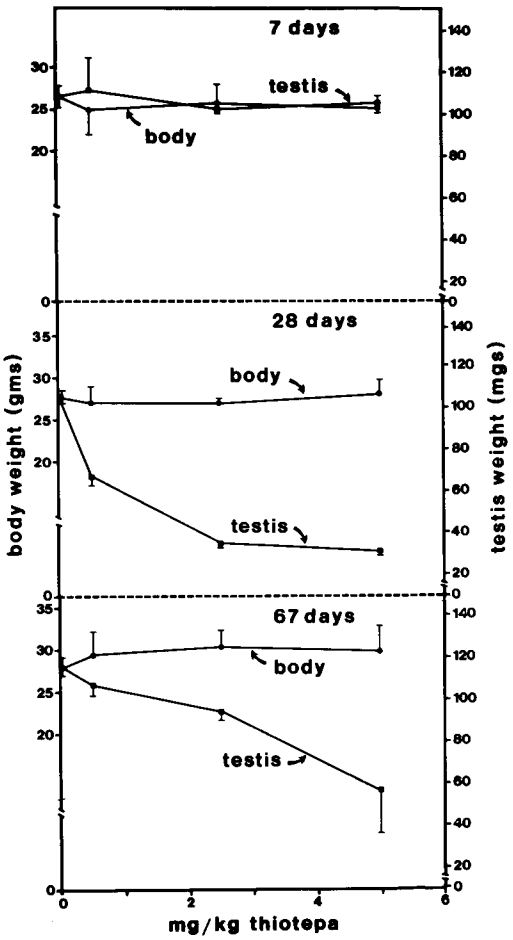

FIG. 1. Response of body and testicular weights to thiotepa exposure (0 to 5.0 mg/kg × 5 days) at 7, 28, and 67 days ALE.

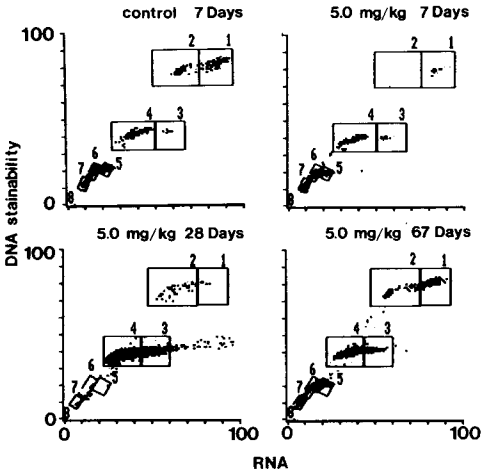

FIG. 2. FCM cytograms showing effect of thiotepa (5 mg/kg × 5 days) on two-parameter (area of green vs area of red fluorescent signal) distribution of TSAO-stained testicular cells obtained from mice at 7, 28, and 67 days ALE. Green and red fluorescence values correspond to DNA and RNA stainability, respectively. Control samples were obtained from animals receiving solvent buffer only.

Testicular cell measurements. Figure 2 demonstrates the effect of 5 mg/kg thiotepa on the FCM distribution of AO-stained testicular cells obtained from a control mouse and mice at 7, 28, and 67 days ALE. Each point on the cytograms represents four or more individual cells. Seven clusters of cell types, each boxed off and numbered, are resolved and correspond to: (1, 2) 4N cells; (3, 4) 2N cells; (5) round spermatids; (6) elongating spermatids; (7) elongated spermatids. Region 8 corresponds to the position occupied by mature sperm. Only box 2 shows an absence of cells at 7 days ALE. At 28 days, boxes 1, 5, 6, and 7 are nearly devoid of cells. Recovery toward normality is seen by Day 67. The relative numbers of each cell type are shown in Fig. 4.

Figure 3 presents the raw data in another

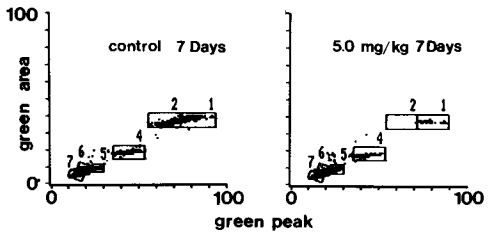

FIG. 3. Effect of thiotepa (5 mg/kg × 5 days) on two-parameter (area of green vs peak of green fluorescent signal) distribution of testicular cells from untreated animals and those at 7 days ALE. The raw data are the same as from Fig. 2.

way in an attempt to further resolve subpopulations. In this case, the green fluorescent signal was electronically processed as area of signal vs peak of signal. The area of the green signal is the area of the total nuclear DNA fluorescent signal integrated over time which is independent of nuclear size. The peak of the green fluorescent DNA signal is dependent on nuclear size. Thus two cells with the same DNA stainability and total fluorescence but differing in nuclear size would have the same area signal; however, the cell with a smaller nucleus would have a higher, narrower peak signal than the one with a large nucleus which would have a low, broad peak signal. This principle allows for discrimination between mono- and binucleated cells and also G_2 phase cells and doublets of G_1 phase cells. As seen in Fig. 3, no additional subpopulations can be detected by this method. The percentages seen in Table 1 of cells in boxes 1 and 2 of Figs. 2 and 3 support the view that they represent the same cells. This table also shows an approximate 70% loss of 4N cells in box 1 relative to the controls 7 days ALE.

Figure 4 shows the relative percentage of testicular cell types present in mouse testes at 7, 28, and 67 days ALE. These values were obtained by computer assistance from raw data obtained as described in Fig. 2. At 7 days ALE to the 0.5 mg/kg dosage, a statistically significant ($\bar{x} > 2$ SD from control level) decrease in percentage of round spermatids and increase in percentage of elongating spermatids was observed. A decreased percentage of 4N cells was observed and a corresponding relative increased percentage of 1N cells. Measurements of 40 control testicular samples in 10 other independent experiments showed a mean of 84.2 ± 1.5% 1N, 7.4 ± 0.9% 2N, 8.4 ± 1.0% 4N. For 1N cell types there were 42.8 ± 3.1% round spermatids, 24.6 ± 3.0% elongating spermatids, and 32.2 ± 3.3% elongated spermatids. Data in Fig. 2 and Table 1 show the drop in 4N cell numbers is from a decrease in box 1 and a total loss of cells in box 2.

Histological observations of sections prepared from mouse testes at 28 days ALE showed that 1N cell types were highly depleted after exposure to 2.5 mg/kg. At 5.0 mg/kg the seminiferous tubules were essentially devoid of all germ cell types except some spermatogonia. Flow cytometric measurements of testicular samples obtained from mice 28 days ALE showed a dramatic alteration in the relative percentage of testicular cell types present as seen in Fig. 4. A dosage of 2.5 mg/kg caused a reduction of 1N cell types from 82 to 4% of total; the relative percentage of diploid cells increased correspondingly from 9 to 72%, and the relative percentage of 4N cell types increased from 9 to 24%. The haploid population was virtually absent at 5.0 mg/kg.

At 67 days ALE testicular populations were recovering from chemical-induced testicular damage. Some effect was still noted by FCM, particularly at the highest dosage, but not as great as at 28 days ALE (Fig. 4). Histology

TABLE 1
Percentage of total
~~CORRELATION BETWEEN~~ CELL POPULATIONS DISTINGUISHED BY TWO DUAL-PARAMETER FCM METHODS

	Control		7-day ALE	
	Box 1	Box 2	Box 1	Box 2
Green area vs red area (Fig. 4)	5.8	3.4	1.4	0
Green area vs green peak (Fig. 5)	5.5	3.4	1.6	0

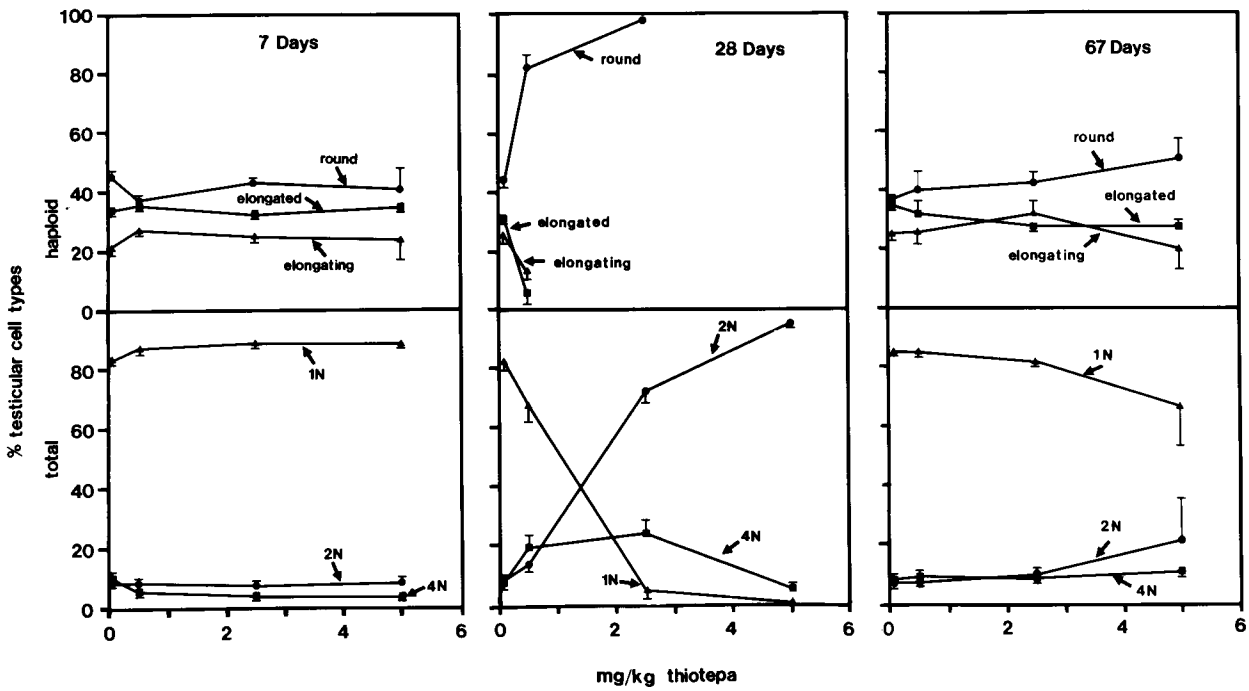

FIG. 4. Effect of various doses of thiotepa on presence of five testicular cell types present 7, 28, and 67 days ALE. Each point represents the mean of three values obtained from randomly chosen individual mice per dosage/time point. The vertical bars shows SDs. The values for round and elongating spermatids 7 days ALE to 0.5 mg/kg are $\geq$ 2 SD from the control values. All values for 4N cell types at 7 days ALE are > 2 SD from control values.

sections of the contralateral testis of the same treated animal showed that about half of the seminiferous tubules were repopulated in mice exposed to 5.0 mg/kg. All tubules were repopulated at the lower dosages.

Figure 5 compares the effects of 5.0 mg/kg on testicular cell types present at 7, 28, and 67 days ALE. These isometric displays demonstrate the loss and recovery of various testicular cell populations.

Other data (not shown) indicated thiotepa was also cytotoxic to the hematopoietic system since peripheral blood leukocytes isolated on Ficoll–Hypaque gradients and stained by TSAO showed an increased percentage (4% from 2.5 mg/kg dosage) in cell cycle relative to control (0.8%).

Effect of Thiotepa on Epididymal Sperm

Figure 6 and Table 2 demonstrate the effect of thiotepa on epididymal sperm head morphology and $\bar{x}\ \alpha_t$, SD α_t, and CV α_t of TSAO-stained sperm and AO-stained, heat-denatured sperm nuclei.

Sperm head morphology. All concentrations of thiotepa used had no significant (<1 SD from control mean) effect on sperm head morphology 7 days ALE. However, 28 days ALE a dosage of 2.5 mg/kg caused a significant increase from 2.7% background to 6.1%, and 5.0 mg/kg caused 7.4% sperm head abnormalities. By 67 days ALE, the dosages of 2.5 mg/kg and 5.0 mg/kg caused an increase of sperm head morphology abnormalities to 8.7 and 11.6%, respectively (Fig. 6).

FCM measurements of sperm chromatin structure. FCM measurements of whole sperm stained by the TSAO technique detected chemically induced alterations in sperm chromatin at the same minimal effective dose as the sperm head morphology assay (Fig. 6). Although the mean values of α_t ($\bar{x}\ \alpha_t$) did not vary, the coefficient of variation and especially standard deviation of α_t (CV α_t and SD α_t, respectively) were sensitive to sperm chromatin alterations when assayed by TSAO staining of whole sperm (Fig. 6). Note the similarity of SD α_t values between measurements of whole sperm by the TSAO method and isolated nuclei by the thermal denaturation method (28 days, Fig. 6).

Correlation coefficients were calculated between sperm morphology and α_t parameters. Regression equations also were generated on the same data with dose as the independent variable. As shown in Table 2, no significant differences in sperm head abnormalities were

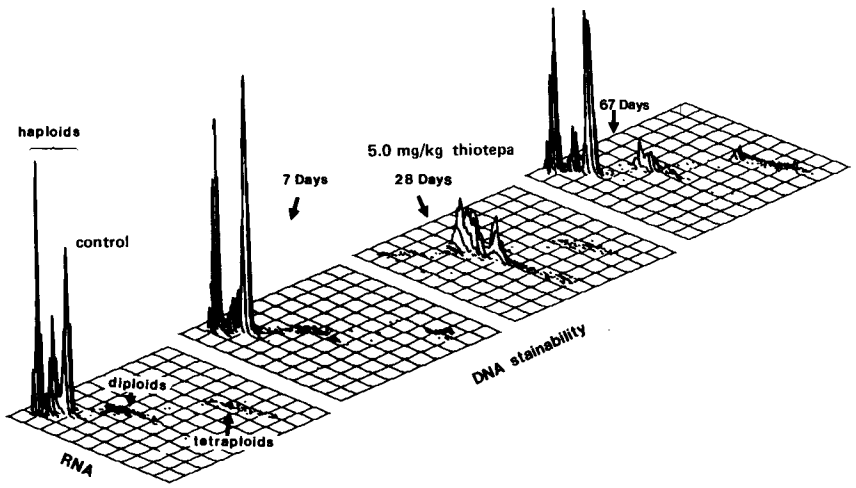

FIG. 5. Computer-generated isometric display of distribution of testicular cells obtained from control and 5.0 mg/kg-treated mice according to their red and green fluorescence at 7, 28, and 67 days ALE.

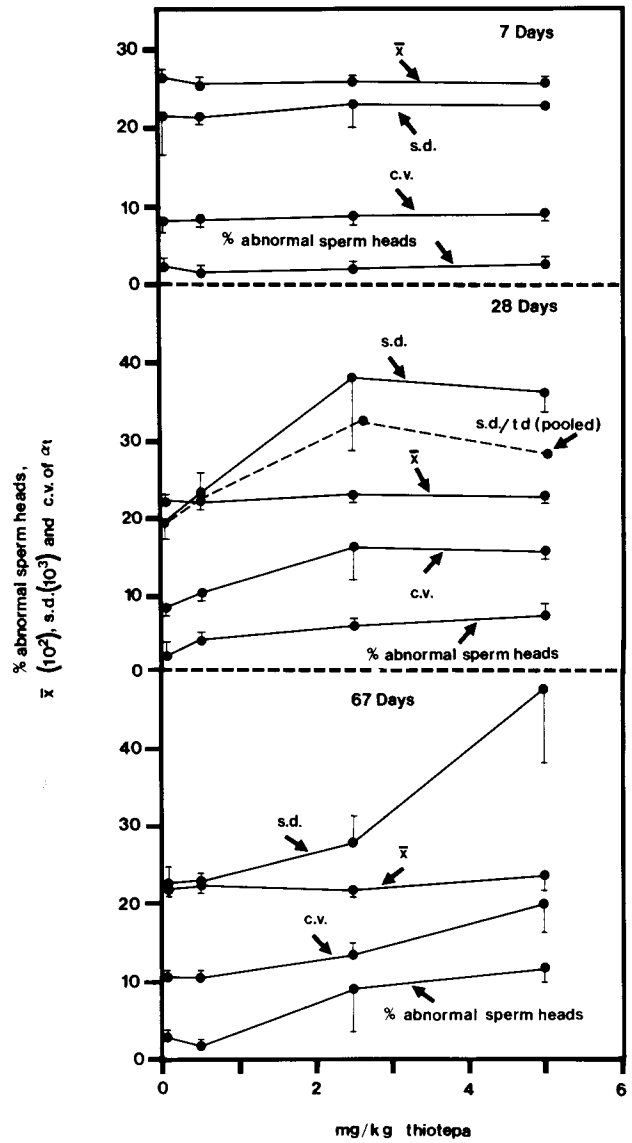

FIG. 6. Effect of thiotepa (0 to 5 mg/kg × 5 days) on epididymal sperm head morphology and $\bar{x}$ α_t, SD α_t, and CV α_t of TSAO-stained sperm at 7, 28, and 67 days. Each point represents computer-derived mean values obtained from at least three individual mice from each time/dosage group. No effect was observed at 7 days. At 28 and 67 days and at 2.5 mg/kg the SD α_t and abnormal morphology points are all $\geqslant$ 1 SD from the control mean; at 5.0 mg/kg the SD α_t is $\geqslant$ 2 SD from the control mean and the abnormal morphology value $\geqslant$ 1 SD from the mean.

detected at 7 days ALE by either FCM or light microscopy, and variables in Table 2 are not related. A test of the slope of the regression lines indicated neither line was different from 0, i.e., there was no dose–response at this time. However, at 28 days ALE, dose–response is evident for both percentage of abnormal heads and CV α_t. At 67 days ALE this is even more apparent and correlations between variables are highly significant ($p < 0.01$). At 28 days

TABLE 2

RELATIONSHIPS BETWEEN EFFECTS OF THIOTEPA ON SPERM HEAD MORPHOLOGY AND CHROMATIN STRUCTURE

	Correlation coefficients				Regression coefficients[a]	
n	Days ALE	Dose with % abnormal	Dose with CVα_t	% Abnormal with CVα_t	% Abnormal	CVα_t
8	7	0.09	0.38	0.61	0.03	0.16
12	28	0.76†	0.78†	0.48*	0.86†	1.46†
14	76	0.86†	0.89†	0.85†	2.04†	1.98†

[a] HO:b = 0, dose = independent variable.
* $p < 0.05$.
† $p < 0.01$.

ALE a high correlation (0.98, $p < 0.05$) existed between SD α_t for measurements of whole sperm stained by the TSAO method, and fixed, thermally denatured, AO-stained nuclei.

Figures 7 and 8 show exposure to thiotepa caused an increased percentage of detached sperm heads in preparations of epididymal sperm, most likely due to lack of attachment and/or increased susceptibility to detachment due to physical stresses that accompany isolation of epididymal sperm. However, other data not presented here showed the same percentage of detached heads in samples whether obtained directly from epididymal fluid without agitation or from minced epididymi prior to TSAO staining and FCM measurement. At 28 days ALE a near doubling in percentage of detached heads was observed for mice treated at 0.5 mg/kg (Fig. 8). Higher dosages did not show a significant difference from control values. At 67 days ALE, the curve shows an increase in percentage of detached heads at 5.0 mg/kg, but this is offset by a large SD, indicating mouse-to-mouse variation in response to this concentration of chemical exposure. The meaning of these differences is not clear, especially because a significant difference was observed between control groups; even so, note the relatively small standard deviations indicating homogeneity among most groups. Of interest, detached heads stained by the TSAO method had a higher CV α_t, indicating DNA in these heads had a greater susceptibility to *in situ* denaturation than did DNA in intact sperm (Fig. 8B).

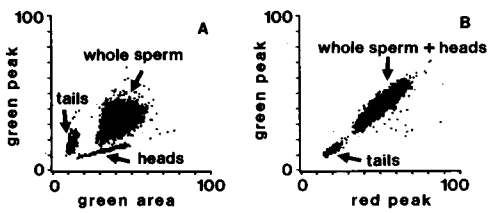

FIG. 7. Distribution of TSAO-stained whole sperm and detached sperm heads when displayed as peak vs area of green fluorescent signal. The clusters were verified by adding TSAO-stained populations of purified mouse sperm heads or tails to the sample. Furthermore, the ratio of free heads to whole sperm seen under a light microscope agreed with the computer-derived values.

DISCUSSION

The rodent male reproductive tract provides a nearly ideal organ system to study acute and chronic effects of drugs and environmental chemicals on cell proliferation and differentiation. Dual-parameter FCM can rapidly and simultaneously measure seven or more testicular cell types and extratesticular sperm at several stages of maturation, thereby providing a powerful system for evaluating *in vivo* toxic effects on cellular systems.

In this study, seven testicular cell types and

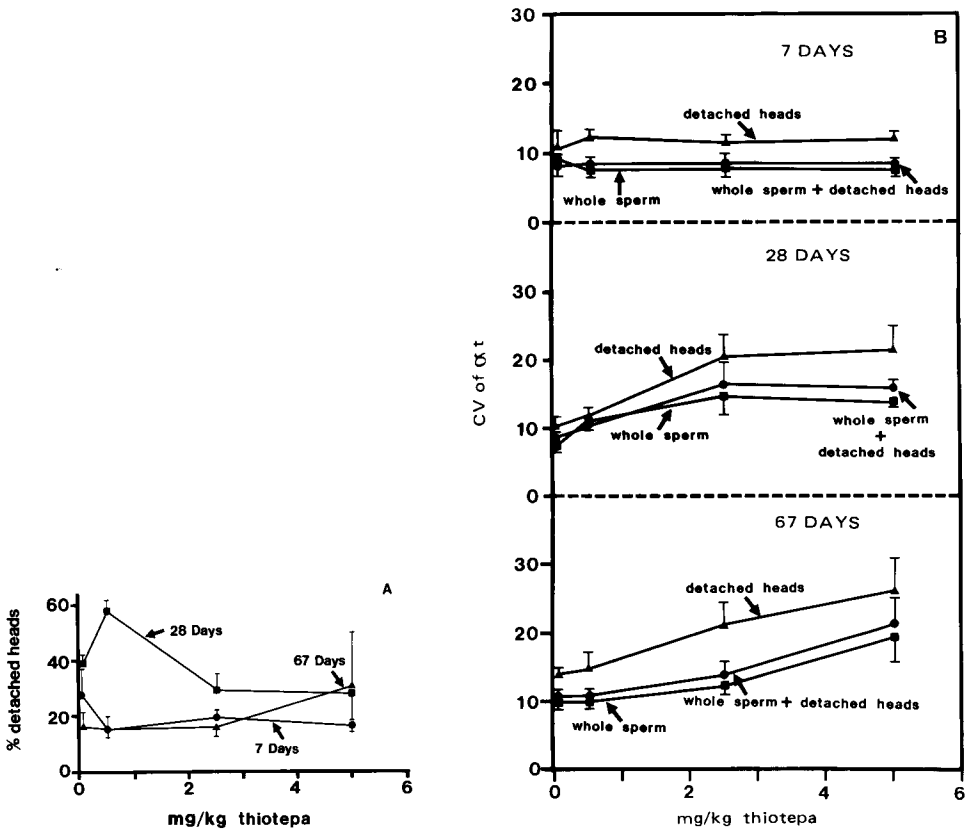

FIG. 8. Effect of various dosages (0 to 5 mg/kg × 5 days) of thiotepa on percentage of detached sperm heads (A) and CV α_t of whole sperm and detached heads (B) in preparations of epididymal sperm from mice 7, 28, and 67 days ALE. Each point represents the mean of three measurements on individual sperm samples from mice exposed to different concentrations of thiotepa.

epididymal sperm were evaluated 7, 28, and 67 days ALE to thiotepa. By 7 days ALE, two detectable events were seen by our measurements of testicular cells (Fig. 2).

First, we noted a total absence of one 4N population of testicular cells (box 2, Figs. 2 and 3) and a reduced number in box 1. The difference measured between boxes 1 and 2 in Fig. 2 is the amount of red fluorescence, i.e., RNA content. The difference measured between boxes 1 and 2 in Fig. 3 is nuclear size. Thus, the cells lost had a lower RNA content but a larger nucleus or more than one nucleus. Future FCM sorting experiments will identify the cell type(s) lost by 7 days ALE, but for now it seems likely the cell type missing is the pachytene primary spermatocyte. This cell has a relatively large nucleus (Perey *et al.*, 1961) but has not yet begun the significant increase of RNA characteristic of late pachytene and diplotene spermatocytes (Courot *et al.*, 1970). Exposure to thiotepa most likely killed the pachytene-stage spermatocyte directly causing the 4N population decrease, as has been previously suggested (Meistrich *et al.*, 1982).

Second, at the lowest concentration used (0.5 mg/kg), a significant relative decrease in percentage of round spermatids and an increase in percentage of elongating spermatids was noted 7 days ALE with no change seen in

percentage of elongated spermatids. If pachytene spermatocytes are the target of thiotepa and most are damaged or killed, the decrease in round spermatid number 7 days later may reflect maturation depletion effects. Similarly, the apparent increase in elongating forms may reflect the continued maturation of round spermatids present prior to thiotepa treatment, as well as the changing distribution of cell types within the treated testis. Thiotepa has been shown (Meistrich *et al.*, 1982) to cause degeneration of cells in the process of meiotic division.

From data presented in Fig. 4, it is not possible in some cases to determine whether there is an absolute increase or decrease in a particular cell type or only a relative change in a population due to an increase or decrease of another population. The lack of cells in box 2 at 7 days ALE (Fig. 2) can, in this case, simply by interpreted as an absolute loss of that cell type. In contrast, the relative increase of 2N cell types at 28 days ALE (Fig. 4) is likely due to an almost complete loss of germ cells with little or no damage to interstitial cells or structure of the tubule as seen in the histology sections. The 2N population contained two groups of cells that differed by RNA content; these cells were not further identified. By 67 days ALE, the testes were recovering from the higher dosages that apparently killed a high percentage of spermatogonial stem cells. Since only half of the tubules were repopulated after exposure at the highest dose, this agrees with previous observations (Meistrich *et al.*, 1982) that thiotepa produces strong stem cell toxicity.

The nearly parallel alterations of sperm head morphology and SD α_t is of significant interest and very similar to that seen in a previous study on the effects of another alkylating agent, ethylnitrosourea (ENU, Evenson *et al.*, 1985). These data suggest that alterations of chromatin structure not seen by light or electron microscopy (Evenson *et al.*, 1985) are detected as an increased susceptibility to DNA denaturation *in situ*. These data also support a suggestion made in the ENU study that acid (TSAO) is as effective to induce partial DNA denaturation *in situ* as the thermal technique originally used (Evenson *et al.*, 1980a). This possibility was not readily appreciated since it was thought the TSAO technique did not denature DNA *in situ*, at least in somatic cells (Darzynkiewicz *et al.*, 1984). However, these and other data (Evenson *et al.*, 1985, 1986) suggest sperm DNA is packaged in a manner allowing acid- or heat-denatured DNA to interact similarly with AO to produce red fluorescence.

Of interest, sperm heads free of tails have a higher CV α_t than whole sperm. Since heads detached from tails may reflect abnormalities of spermatogenesis, chromatin in these heads may also be abnormal. For example, disulfide bonding of both nuclear chromatin protamines and other sperm structures, including the connection between the sperm head and midpiece, occurs during spermatogenesis; thus a defect in disulfide bonding could affect both parameters. Mouse sperm chromatin condensation progresses from nuclear anterior to posterior (Evenson *et al.*, 1984) and if the process were incomplete, posterior-located chromatin would be expected to denature more easily. Fluorescence microscopy of AO-stained abnormal sperm supports this view (Evenson *et al.*, 1980a). It is not clear why an intermediate dosage of thiotepa had a positive effect in percentage of detached heads while a higher dose showed no change; however, it is likely that cell kill and maturation depletion are related to the observation.

This flow cytometry study used several dual-parameter combinations to elucidate thiotepa-induced cellular alterations. These included: (1) DNA vs RNA content; (2) ratio of double-stranded vs single-stranded DNA; and (3) area vs peak of DNA fluorescent signal. These combinations of dual parameters demonstrate the power of flow cytometry for toxicologic studies, including the ability to follow toxin-altered kinetics of seven testicular cell types. Of particular interest is the observation that

our sperm chromatin structure assay detects dose-response at the same minimal effective dose as the sperm head morphology assay.

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