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Induction of spermatogonial stem-cell damage by chloral hydrate

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**INDUCTION OF SPERMATOGONIAL STEM-CELL DAMAGE BY
CHLORAL HYDRATE**

A Thesis

Presented to

The Faculty of the Department of Biological Sciences
San Jose State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts

by

Elizabeth Ann Villalobos Nutley

December, 1994

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ABSTRACT

INDUCTION OF SPERMATOGENIAL STEM-CELL DAMAGE BY CHLORAL HYDRATE

by Elizabeth Ann Villalobos Nutley

Spermatid micronuclei (SMN) were investigated in male B6C3F1 mice after injections of the anesthetic, chloral hydrate (CH; 2,2,2-trichloro-1,1-ethanediol), during three periods of spermatogenesis. SMN were evaluated on coded slides independently by two laboratories. Both laboratories found increased frequencies of SMN after administration of CH to spermatogonial stem cells but not to spermatocytes in early or late meiosis. Whole chromosome malsegregation into SMN was evaluated by kinetochore immunofluorescence and fluorescence *in situ* hybridization (FISH) with a pan-centromeric DNA probe. One laboratory observed elevated frequencies of kinetochore-positive (K+) SMN at CH doses of 82.7 and 413.5 mg/kg after stem-cell treatment. The other laboratory found dose-related increases in frequencies of pan-centromeric labeled (PC+) SMN following stem-cell treatment. These results suggest a moderate aneuploidogenic action of CH on spermatogenic stem cells of mice.

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DEDICATION

This paper is dedicated to my family and friends for their confidence in me and the love they have shown me. It is particularly dedicated to my husband, Dale, and to my son, Calvin, who have been the incentives for me to return to college and have supported my efforts so well and for so long.

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INTRODUCTION

Chloral hydrate (CH; 2,2,2-Trichloro-1,1-ethanediol; Merck Index, 1983) is a hypnotic drug that is used as a mild sedative for patients of all ages (Manual of Medical Therapeutics, 1980). It is the most commonly used sedative for young children and infants undergoing diagnostic procedures in hospital pediatrics units and is given at doses of 25 to 100 mg/kg (Cook, 1992). CH is also a metabolite of the common solvent trichloroethylene (TCE; Waters et al., 1977).

Chronic exposure to CH induced hepatocarcinomas in male B6C3F1 mice (Daniel et al., 1992), but it is only a weak mutagen *in vivo*, and of variable mutagenicity *in vitro*. CH was directly mutagenic in *Salmonella typhimurium* TA100 (Waskell, 1978; Bignami et al., 1980; Haworth et al., 1983; Fu et al., 1994), and induced base-pair substitutions in the filamentous bacterium *Streptomyces coelicolor* (Bignami et al., 1980). It weakly induced point mutations in the fungus *Aspergillus nidulans* (Bignami et al., 1980) and gene conversion in the yeast *Saccharomyces cerevisiae* (Bronzetti et al., 1984). CH binding to mammalian DNA *in vivo* and *in vitro* was negligible (Fu et al., 1994), and CH failed to cause DNA strand breaks *in vivo* in rodent liver in one investigation (Chang et al., 1992) but caused DNA strand breaks in an earlier investigation (Nelson and Bull, 1988) and it caused lipid peroxidation *in vitro* (Fu et al., 1994). Induction of chromosome aberrations by CH in mouse cells *in vivo* is equivocal. Russo et al. (1984) found a significant induction of chromosome damage in spermatocytes, but insignificant effects were reported in later investigations of bone marrow cells (Xu and Adler, 1990) and spermatocytes (Russo and Levis, 1992b).

CH alters the functioning of spindle microtubules in many test systems. It caused inhibition of *in vitro* tubulin assembly in cell-free isolates of porcine

microtubules (Brunner et al., 1991) and bovine microtubules (Wallin and Hartley-Asp, 1993), and caused chromosome malsegregation in *S. cerevisiae* (Albertini, 1990). It induced the disappearance of mitotic spindles in *A. nidulans* (Mercer and Morris, 1975), in plant endosperm (Molè-Bajer, 1969), and in eggs of *Pleuordeles waltii* (Sentein and Ates, 1974), and blocked spindle formation in grasshopper spermatocytes (Ris, 1949). It caused abnormalities in chromosome division in Chinese hamster cells *in vitro*, including loss of the mitotic spindle, hypodiploidy and tetraploidy (Warr et al., 1993). C-mitosis was evident in mouse bone marrow cells treated with CH *in vivo* (Miller and Adler, 1989). CH induced aneuploidy in evolutionarily diverse species, including *A. nidulans* (Singh and Sinha, 1976; Crebelli et al., 1985; Käfer, 1986; Parry, 1993), *S. cerevisiae* (Parry, 1993), Chinese hamster cells *in vitro* (Natarajan et al., 1993), and human lymphocytes *in vitro* (Vagnarelli et al., 1990; Sbrana et al., 1993). In mice, CH induced aneuploidy in polychromatic erythrocytes (PCEs; Leopardi et al., 1993). Results in spermatocytes have been variable, with significant induction of hyperhaploidy in some studies (Russo et al., 1984; Liang and Pacchierotti, 1988; Miller and Adler, 1992) but not others (Leopardi et al., 1993).

Micronucleus (MN) assays in somatic cells are frequently applied to the study of induced chromosomal damage (Heddle et al., 1991). MN may arise during cell division by failure of whole chromosomes to segregate properly in mitosis or meiosis or from breakage of chromosomes into acentric fragments. The adaptation of these assays to use in the germ line provides a method of screening chemical and physical agents for ability to cause reproductive abnormalities. The use of a spermatid micronucleus (SMN) assay for detecting clastogenic action or aneuploidies in germ cells of mice was proposed by Lähdetie and Parvinen (1981) and by Tates et al. (1983). Clastogenic agents that

have induced SMN in mice include X-rays, fast fission neutrons, adriamycin, cyclophosphamide, dimethylbenz[a]anthracene, ethyl methanesulfonate, ethylnitrosourea, mitomycin C (MMC) and methyl methanesulfonate (for review, Russo and Levis, 1991). The frequencies of SMN in mice increased following CH treatment of spermatocytes which were in diakinesis through meiotic metaphase II (Russo and Levis, 1992b) and after treatment of spermatogenic stem cells (Allen et al., 1994) but not with treatment of preleptotene spermatocytes (Russo and Levis, 1992a), consistent with CH activity upon spindle microtubules during mitotic and meiotic divisions rather than S-dependent clastogenicity.

Two current methods for characterizing the cytogenetic lesions within MN are immunofluorescence labeling of kinetochore proteins, and fluorescence *in situ* hybridization (FISH) of centromeric DNA. The presence of centromeric DNA or kinetochore protein in an MN indicates that it contains a centromere and may contain a whole chromosome, while the absence of these markers indicates that the MN formed as a result of chromosome breakage. In mice, FISH analysis has been used to distinguish MN of aneugenic versus clastogenic origin in somatic cells (Hayashi et al., 1994) and in spermatids (Kallio and Lähdetie, 1993). In germ cells, the aneugens vinblastine and etoposide generated greater frequencies of SMN with FISH to centromeric DNA than did the clastogen MMC (Kallio and Lähdetie, 1993). Kinetochore-labeling of MN also differentiated clastogenic from aneugenic effects of several agents, including CH, in mouse somatic cells (Gudi et al., 1992). Kinetochore-labeling has recently been applied to study SMN induced by acrylamide, X-rays (Collins et al., 1992), TCE and CH (Allen et al., 1994). Both TCE and CH induced small but not statistically significant increases in kinetochore-positive SMN (Allen et al., 1994). Simultaneous application of kinetochore-labeling and centromeric DNA analysis

in micronuclei has been used in cytochalasin-B blocked human lymphocytes in culture (Norppa et al., 1993), and in mouse erythrocytes (Miller et al., 1991; Chen et al., 1994).

In the current study, the mechanism of CH action on meiotic and stem cells of male germ cells was investigated using the SMN assay. Slides were coded and SMN scoring was performed independently by two laboratories, including one that had made the initial finding of a stem-cell effect upon SMN (Allen et al., 1994). Two methods were used to characterize the contents of SMN: (a) hybridization with DNA probes specific for centromeric DNA and (b) immunofluorescence staining for the presence of kinetochores. In addition to the centromeric labeling, an X-probe was utilized as an internal check on hybridization efficiency.

MATERIALS AND METHODS

Chloral hydrate

Chloral hydrate (CH; CAS#302-17-0) was obtained from Dr. Jack Bishop at the National Institute of Environmental Health Sciences, Research Triangle Park, NC. Three months before the study, the purity of the compound was confirmed to be 97% by capillary gas chromatography. Dilutions were made using sterile and double-distilled water.

Animal conditions and treatment

Male B6C3F1 mice (Simonsen Farms, Gilroy, CA), 10 to 13 weeks of age at treatment, were housed in the Lawrence Livermore National Laboratory (LLNL) Animal Care Facility, given sterile tap water and Purina Certified Rodent Feed #5002 (Dean's Animal Feed, Belmont, CA) *ad libitum*, and kept on a 12-hour light/dark cycle in a ventilated room at 24°C. For up to one week following CH administration, the mice were housed individually in laminar flow units to decrease the chance of cross-contamination between treatment groups.

Following random assignment to CH dosage groups, mice received single intraperitoneal (i.p.) injections of CH at concentrations of 0.0, 82.7, 165.4, or 413.5 mg/kg body weight. Spermatids were harvested from 4 to 6 mice per dose group at 4-5, 14-15 and 40-41 days after treatment. These intervals correspond to cell treatment in pachytene-diakinesis, preleptotene and spermatogonial stem cell stages (Oakberg, 1957). On the day of sacrifice, cage cards were recoded to blind technicians to the dosage group to which each mouse belonged and mice were then sacrificed by CO₂ asphyxiation.

Cell preparation

The spermatid preparations were performed at Laboratory 2 (LLNL) using the method of Bates (1983) with modifications. Tunicae were removed from both testes, then the tubules gently teased apart in testis isolation medium [TIM; per 10 liter: NaCl (60.5 g), KCl (33.9 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.8 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3.0 g), Na_2HPO_4 (8.5 g), KH_2PO_4 (0.9 g), glucose (10 g), pH 7.2-7.3; Dietrich et al., 1983]. Cell suspensions were incubated with collagenase (2.5 mg/testis; Type 1A, Sigma Chemical, St. Louis, MO), then trypsin (2.5 mg/testis, Gibco BRL, Gaithersburg, MD), washed 2X in TIM, then filtered through nylon mesh. Following another wash in TIM, one drop of the suspension was placed on each pre-cleaned slide. The slides were air-dried, baked at 80°C for 30 min, cooled to room temperature, and treated for 1 min with 0.5% Tween-20 in phosphate-buffered saline. The slides were dehydrated in an ethanol series (50, 70, 85 and 100%; 2 min each) and stored at -20°C in N₂-flushed polyethylene bags. A subset of coded slides was express mailed on dry ice to collaborators at Laboratory 1 (United States Environmental Protection Agency; EPA).

Laboratory 1: Kinetochore labeling and scoring

Slides were stained in Laboratory 1 using the SMN kinetochore immunofluorescence procedure (Allen et al., 1994). Briefly, slides were pre-rinsed with 0.3% Tween-20 (Sigma Chemical Co., St. Louis, MO), incubated with anti-kinetochore antibody (CREST antibody; Chemicon, Inc., Temecula, CA), washed again with Tween-20, and incubated with fluoresceinated goat anti-human antibody (Chemicon, Inc., Temecula, CA). Slides received a final rinse with Tween-20 followed by application of 1.5µg/mL DAPI (4,6-diamidino-2-phenylindole; Sigma Chemical Co., St. Louis, MO) in antifade (Johnson and de

C. Nogueira Araujo, 1981).

Coded slides were scored with a Leitz fluorescence microscope as described by Allen et al. (1994). Simultaneous phase contrast microscopy and DAPI excitation were used to score round, early-step spermatids and micronuclei. Fluorescein filters were used to detect kinetochores in DAPI-positive micronuclei, and micronuclei were categorized as either DAPI-positive only or DAPI and kinetochore-positive (K+ SMN). A total of 2,000 cells per mouse (4,000 to 6,000 per dose) was scored.

Laboratory 2: DNA probe preparation, fluorescence *in situ* hybridization, and scoring

Laboratory 2 scored slides for the fraction of DAPI-positive SMN and characterized the SMN using FISH. Two DNA probes were used for FISH: DXWas-70, an X-specific repetitive DNA sequence that was a gift from Dr. Christine Disteché (Disteché et al., 1985), and mouse Cot-1™ DNA, a repetitive sequence specific to mouse minor satellite DNA (Gibco BRL, Gaithersburg, MD). The clone for the chromosome X probe was transformed in *E. coli* DH5a™ (Maximum Efficiency DH5a™ competent cells, Gibco BRL) and the plasmid preparation made with a Qiagen Plasmid Kit (Qiagen Inc., Chatsworth, CA). The probes were labeled through nick translation (Nick Translation System, Gibco BRL). The chromosome X probes were labeled with biotin-14-dATP (Gibco BRL) and the pan-centromeric Cot-1™ probe with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN).

Hybridizations were carried out as described by Wyrobek et al. (1994) with some modifications. Briefly, the labeled chromosome X and pan-centromeric probes were suspended in Master Mix 2.1 (55% formamide/10%

dextran/2XSSC) and denatured for 5 min at 70°C. Slides were pretreated for 5 min with 1% RNase (Boehringer Mannheim, Indianapolis, IN) in 2XSSC and then washed for 5 min in PN buffer (NaHPO₄, NaH₂PO₄ and Nonidet P-40 detergent). Slides were then denatured for 4 min at 70°C and then dehydrated in an ethanol series (70, 85 and 100%; 2 min each). Slides with probe were incubated at 37°C overnight, then washed three times in 45°C washing solution (50% formamide, 2XSSC), two times in 45°C 2XSSC, and twice in room temperature PN buffer for 10 min each.

Digoxigenin-labeled pan-centromeric probe was detected with anti-digoxigenin antibody conjugated with rhodamine and the biotinylated X probe with Avidin D conjugated to FITC as described by Wyrobek et al. (1994). DAPI (0.5µg/mL; Sigma Chemical Co., St. Louis, MO) in antifade (Vectashield Mounting Medium, Vector Labs, Burlingame, CA) was used to counterstain the spermatid DNA.

Slides were scored with a Zeiss fluorescent microscope as described by Wyrobek et al. (1994). The filter packages allowed detection of two hybridization colors plus the counterstain simultaneously (red, green, and blue) or individually (blue alone, red plus green alone, red alone, or green alone). First, the coded slides were examined for the presence of round spermatids and micronuclei using the DAPI filter set-up (Zeiss filter 487901). To be scored, a micronucleus had to be clearly separated from the cell nucleus and lack a "stalk" of DAPI-positive material connecting it to the main nucleus (Figure 1).

Figure 1. Micronucleated round spermatid of a mouse treated with chloral hydrate under fluorescence microscopy. Nuclear material of the main nucleus and the micronucleus are stained blue with DAPI counterstain with no "stalk" connecting the two, showing that the micronucleus is distinct from the main nucleus (by A.C.Tcheong. Used with permission).

Micronuclei were scored for the presence of the X-chromosome probe (green) and pan-centromeric probe (red; Figures 2, 3). Micronuclei were classified by three fluorescent phenotypes: DAPI-positive only, DAPI and X-positive (X+ SMN), or DAPI and pan-centromeric-positive (PC+ SMN). A total of 6,000 cells was scored per dose of CH (2,000 to 3,000 per animal; Table I). Scoring was tabulated with the assistance of CYTOscore I, a Macintosh-based program developed at LLNL.

Statistical analyses

Cochran's test of equal proportions (Snedecor and Cochran, 1980a) was used to evaluate the significance of differences in micronucleus frequencies observed between control and treatment doses. A test for linear trend in proportions was performed to analyze dose response (Snedecor and Cochran, 1980b).

Figure 2. A pan-centromeric DNA positive micronucleated spermatid seen under DAPI illumination. The same cell is shown in Fig.3 using the triple bandpass filter combination (by A.C.Tcheong. Used with permission).

Figure 3. The same micronucleated spermatid from Fig.2, seen here with a triple bandpass filter combination, revealing rhodamine fluorescence of the pan-centromeric DNA probe hybridized in the main nucleus and in the micronucleus (by A.C.Tcheong. Used with permission).

RESULTS

The effects of CH were evaluated in mice sacrificed at three time points after a single i.p. injection, representing spermatids that were stem cells or in meiosis (early or late) during treatment. Animals were dosed and slides scored as described in Table I. Coded slides were analyzed by two laboratories independently (Laboratory 1 and Laboratory 2), both of which used DAPI fluorescence to detect SMN. The contents of SMN were characterized using either kinetochore-labeling (Laboratory 1 only) or FISH (Laboratory 2 only). Neither laboratory found a spermatid with more than one micronucleus.

No significant differences were observed among untreated animals within each laboratory. Values for all untreated animals were pooled within each laboratory and statistical analyses of treatment effects were performed using within-laboratory pooled control values. Pooled levels of SMN in untreated animals were not significantly different between the two laboratories (Laboratory 1, 0.5 per 1,000, Table II; Laboratory 2, 1.1 per 1,000, Table III).

SMN after treatment of stem cells

Laboratory 1 reported a significant increases in SMN frequency when all animals in the stem-cell treatment group were compared to pooled controls ($p < 0.025$; Table II). The average increase among treated animals was approximately 3 times the control level, but Laboratory 1 found no evidence of a dose response. However, significant increases were found at individual dose levels of 82.7 ($p < 0.025$) and 413.5 mg/kg ($p < 0.025$).

Table I. Study design and scoring strategy

Cell type treated	Time interval between treatment and sacrifice (days)	Dose (mg/kg)	Laboratory 1			Laboratory 2		
			DAPI and K analyses ^a		Number of mice scored	DAPI, X and PC analyses ^a		Number of mice scored
			Cells per mouse (avg)			Cells per mouse (avg)		
Stem Cell	40, 41	0	2000		3	2167		3
		82.7	2000		3	2117		3
		165.4	2000		3	2033		3
		413.5	2000		3	1925		3
Preleptotene	14, 15	0	2000		2	2000		2
		82.7	2000		2	2000		2
		165.4	2000		2	2000		2
		413.5	2000		2	2000		2
Diakinesis	4, 5	0	2000		2	b		—
		82.7	2000		2	—		—
		165.4	2000		2	—		—
		413.5	1500		2	—		—

^a Laboratories 1 and 2 both used DAPI to detect micronuclei. Kinetochore (K) immunofluorescence was used to characterize spermatid micronuclei by B.W.Collins and J.W.Allen of Laboratory 1. FISH using DNA probes specific for the X chromosome and pan-centromeric DNA (X and PC) was used to characterize spermatid micronuclei by A.C.Tcheong of Laboratory 2.

^b not scored.

Table II. Detection of murine SMN^a and characterization with kinetochore immunofluorescence by Laboratory 1b

Cell type treated	CH dose (mg/kg)	Frequencies of SMNC			Frequencies of K+ SMND			Percent K+ SMNe
		raw number per mouse	freq. per 1,000		raw number per mouse	freq. per 1,000		
Stem Cell	0	0,1,1	0.3		0,0,0	0.0		0
	82.7	3,4,4	1.8 ^f		0,0,2	0.39		18
	165.4	2,2,3	1.2		0,0,1	0.2		14
	413.5	2,3,4	1.5 ^f		0,1,1	0.39		22
Preleptotene	0	0,2	0.5		0,0	0.0		0
	82.7	0,1	0.3		0,0	0.0		0
	165.4	0,0	0.0		0,0	0.0		0
	413.5	1,2	0.8		0,0	0.0		0
Diakinesis	0	1,2	0.8		0,0	0.0		0
	82.7	0,0	0.0		0,0	0.0		0
	165.4	0,0	0.0		0,0	0.0		0
	413.5	2,2	0.8		0,1	0.2		25

Scoring was conducted by B.W.Collins and J.W.Allen. Data used with permission.

^a Spermatid micronuclei.

^b Numbers of mice and cells scored per mouse are described in Table I.

^c For Laboratory 1, the pooled SMN frequency among control mice of 0.5 per 1,000 cells was used for statistical analyses.

^d Kinetochore-positive SMN.

^e % = (number of K+ SMN)/(total number of SMN)X100.

^f p<0.025

^g p<0.05

Table III. Detection of murine SMN^a and characterization by FISH with probes for the X chromosome and centromeric DNA by Laboratory 2^b

Cell type treated	CH dose (mg/kg)	Frequencies of SMN ^c			Frequencies of PC+ SMN ^d			Percent PC+ SMN ^e
		raw number per mouse	freq. per 1,000	raw number per mouse	freq. per 1,000	raw number per mouse	freq. per 1,000	
Stem Cell	0	2,3,3	1.2	0,0,0	0.0	0,0,0	0.0	0
	82.7	1,2,2	0.8	0,0,1	0.2	0,0,1	0.2	20
	165.4	4,6,8	3.0 ^g	0,0,2	0.3 ^f	0,0,2	0.3 ^f	11
	413.5	3,4,7	2.4 ^f	2,2,2	1.0 ^h	2,2,2	1.0 ^h	43
Preleptotene	0	2,4	1.0	0,0	0.0	0,0	0.0	0
	82.7	5,5	1.7	0,0	0.0	0,0	0.0	0
	165.4	3,4	1.2	0,0	0.0	0,0	0.0	0
	413.5	2,3	0.8	0,0	0.0	0,0	0.0	0

Scoring was conducted by A.C.Tcheong. Data used with permission.

^a Spermatid micronuclei.

^b Numbers of mice and cells scored per mouse are described in Table I.

^c For Laboratory 2, the pooled SMN frequency among control mice of 1.1 per 1,000 cells was used for statistical analyses.

^d Pan-centromeric DNA-positive SMN.

^e % = (number of PC+ SMN)/(total number of SMN)X100.

^f p<0.05

^g p<0.025

^h p<0.001

Laboratory 2 reported a dose-response increase in frequency of SMN following stem-cell administration of CH ($p<0.025$; Table III), with significant increases in SMN frequencies at CH doses of 165.4 mg/kg ($p<0.025$) and 413.5 mg/kg ($p<0.05$).

Laboratory 1 evaluated individual SMN using anti-kinetochore immunofluorescence. There was a significant increase in the average frequency of kinetochore-positive SMN (K+ SMN; $p=0.05$) in the stem-cell treatment group, although there was no significant trend with dose. The K+ SMN frequencies were significantly elevated for the individual dose levels of 82.7 and 413.5 mg/kg when compared with pooled controls ($p<0.05$).

Laboratory 2 found a significant average increase in the frequency of SMN hybridizing with the pan-centromeric probe (PC+ SMN; $p<0.025$) when values for all treated mice were compared with pooled controls, and a statistically significant dose-response was observed ($p<0.025$). Significant increases in PC+ SMN were detected at doses of 165.4 mg/kg ($p<0.05$) and 413.5 mg/kg ($p<0.001$).

Each laboratory reported increases in SMN that lacked kinetochore- or pan-centromeric-labeling following stem-cell administration of CH. Laboratory 1 found an increase in kinetochore-negative SMN (K- SMN) compared to pooled controls with CH at 82.7 mg/kg ($p<0.05$) while Laboratory 2 found an increase in pan-centromeric-negative SMN (PC- SMN) with CH at 413.5 mg/kg ($p<0.05$).

Laboratory 2 employed hybridization with a DNA probe specific for the X chromosome as a control on hybridization and demonstrated high efficiency and reliable hybridization of the main spermatid nuclei with this probe (mean $50.1\pm1.4\%$). No SMN showed hybridization with the X probe, suggesting no preferential inclusion of X-chromosomes in SMN after CH treatment.

SMN after treatment of meiotic cells

Neither lab found evidence of an increase in the frequency of SMN after CH administration during early or late meiosis (Tables II and III). Laboratory 1 found no kinetochore protein-labeled SMN after treatment of early meiotic cells. The investigation of SMN after treatment of late meiotic cells revealed only one K+ SMN (of 7 micronuclei found) with CH at 413.5 mg/kg. Laboratory 2 did not report any SMN with FISH labeling of centromeres.

DISCUSSION

Micronuclei may result from malsegregation of whole chromosomes or from loss of acentric chromosomal fragments during anaphase (Heddle et al., 1991). In this investigation, SMN have been analyzed simultaneously by fluorescence *in situ* hybridization to pan-centromeric DNA and by anti-kinetochore immunofluorescence following *in vivo* administration of CH to male mice. The mouse minor satellite DNA probe specifically hybridizes to a region at or immediately adjacent to the centromere (Broccoli et al., 1990). Auto-antibodies from patients with the CREST variant of scleroderma bind to kinetochore proteins (CREST: calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia; Earnshaw et al., 1986). Pan-centromeric or kinetochore label within a SMN is consistent with the presence of a malsegregated whole chromosome. A SMN lacking both labels is presumed to contain an acentric fragment which arose via chromosome breakage. We have demonstrated that CH treatment of spermatogenic stem cells resulted in the induction of SMN and that a significant proportion of induced SMN contained kinetochore label or pan-centromeric DNA label.

SMN in untreated mice

The baseline values of SMN in control mice reported here compare favorably with literature values for both germinal (Table IV) and somatic cells. The pooled baseline values for total spontaneously occurring SMN in mice in this study corroborate control values previously reported by Laboratory 1 of 0.6 to 1.8 SMN per 1,000 spermatids (Allen et al., 1994) and of 1.2 to 4.0 SMN per 1,000 (Collins

Table IV. Comparisons of frequencies of SMN, K+ SMN, and PC+ SMN among untreated male mice used in various studies

Source	# Mice	Total cells scored	Freq. of SMN (per 1,000)	K+ SMN (%)	PC+ SMN (%)
<i>SMN assays with kinetochore immunofluorescence</i>					
Current study (Lab 1)	7	14,000	0.5±0.4	0.0	.c
Allen et al., 1994	6	6,000 ^d	1.8±0.8	0.0	-
	5	5,000	0.6±0.9	0.2	-
	5	5,000	0.6±0.6	0.2	-
	5	5,000	1.4±1.5	0.2	-
	5	5,000	0.6±0.6	0.0	-
Collins et al., 1992	2	2,000	2.0±0.0	0.0	-
	2	2,000	2.5±0.7	0.0	-
	5	5,000	1.2±0.8	0.0	-
	5	5,000	4.4±4.0	0.6	-
	5	5,000	4.0±0.7	0.0	-
<i>SMN assays with FISH to centromeric DNA probes</i>					
Current study (Lab 2)	5	10,500	1.1±0.3	-	0.0 ^e
Kallio and Lähdetie, 1993	6	31,000	1.4±0.1	-	24.0 ^f
<i>SMN assays without centromeric label</i>					
Russo and Levis, 1992b	3	6,201	0.8±0.4	-	-
	3	6,201	0.5±0.3	-	-
Russo and Levis, 1992a	4	4,115	1.0±0.5	-	-
Lähdetie, 1988	4	4,000	2.0±0.7	-	-
	7	7,000	1.6±0.6	-	-

^a SMN: Spermatid micronuclei; K+ SMN: Kinetochore-positive SMN, using kinetochore immunofluorescence; PC+ SMN: Centromeric DNA-positive SMN, using FISH.

^b Mean ± standard deviation.

^c Dash indicates that the assay was not performed.

^d Separate lines refer to controls from different experiments.

^e FISH using a mouse minor satellite DNA probe.

^f FISH using a mouse major satellite DNA probe.

et al., 1992). These values are consistent with published frequencies of 1.6 to 2.0 per 1,000 (Lähdetie, 1988), 1.0 per 1,000 (Russo and Levis, 1992a), and 1.4 per 1,000 (Kallio and Lähdetie, 1993). Further, the incidence of spontaneously occurring MN in the mouse bone marrow PCE assay has a historical mean of 1.88 MN per 1,000 PCEs (reviewed by Salamone and Mavournin, 1994).

No centromere- or kinetochore-positive micronuclei were identified in vehicle control mice. This is consistent with the previously reported K+SMN rates from Laboratory 1 of 0.0 to 0.6% (Collins et al., 1992) and 0% (Allen et al., 1994), but lower than the 24% centromeric DNA-positive SMN reported by Kallio and Lähdetie using FISH to the mouse major satellite DNA region (1993). Variations among mouse strains, spermatid preparations or in choice of DNA probes may account for these differences. Kallio and Lähdetie applied microdissection of seminiferous tubules to limit their analyses to very early step spermatids, while Labs 1 and 2 used a cell suspension technique adapted from Tates et al. (1983) which included all round spermatids. If SMN were lost from spermatids during very early spermiogenesis, then scoring all round spermatids would reduce the apparent SMN frequencies. However, this is unlikely because consistent levels of SMN were found when SMN were simultaneously assayed in early round spermatids and in late round spermatids (Russo and Levis, 1992b), suggesting that loss of SMN did not occur during spermiogenesis.

The biology of the DNA probes chosen may explain the elevated baseline frequencies observed by Kallio and Lähdetie (1993). Kallio and Lähdetie used the mouse major satellite DNA, which is composed of repeats of a 234 basepair (bp) unit within the mouse constitutive heterochromatin (Moens and Pearlman, 1990). Breakpoints in constitutive heterochromatin have been observed in late

spermatids of mice (Schrierer-Schwemmer and Adler, 1993) and in human lymphocytes (Eastmond et al., 1994), creating acentric fragments which hybridized to major (gamma) satellite DNA probes. Higher levels of "centromere-positive" SMN reported by Kallio and Lähdetie (1993) may include such major satellite DNA-bearing acentric fragments. Laboratory 2 used the mouse minor satellite DNA, which is a 120 bp repeating DNA unit at or immediately adjacent to the kinetochore of each mouse chromosome except for the Y (Broccoli et al., 1990; Wong and Rattner, 1988). The minor satellite is not known to be sensitive to breakage which may explain the low levels of label-positive SMN found in the current study.

The literature frequencies of centromere or kinetochore label in MN of mouse somatic cells are typically higher than those reported for the germinal SMN. Reported rates of centromere-positive MN utilizing FISH with major satellite DNA probes were 42% in mouse PCEs (Miller et al., 1991), 18% in mouse PCEs and 67% in mouse splenocytes *in vivo* (Chen et al., 1994), 30% in mouse fibroblasts *in vitro* (Miller et al., 1992), 29% in a mouse hepatocyte cell line (Salassidis et al., 1992), and ranged from 42 to 55% in isolated MN from peripheral blood of mice (Hayashi et al., 1994). Reported values for K+ MN in mouse PCEs were 0% (of only 2 observed MN; Gudi et al., 1992), 30% (Miller et al., 1991; Miller and Adler, 1990) and 45% (Russo et al., 1992). Kallio and Lähdetie (1993) suggested that a difference in the rate of spontaneous frequencies of centromeric-labeled MN in germinal and somatic cells may be due to vulnerability to damage of the meiotic exchange process. However, it is not possible to exclude technical explanations and studies are needed in which somatic cells and germ cells are analyzed simultaneously with the same labeling procedure.

Induced SMN

Significant induction of SMN by CH was found after stem-cell treatment. The frequencies corroborate the 5.4X increase and dose-response previously reported by Laboratory 1 (Allen et al., 1994). Further, significant elevations were found in frequencies of SMN labeled by kinetochore immunofluorescence or pan-centromeric DNA probe, strongly suggesting the involvement of whole chromosome malsegregation. This finding is consistent with the reported increase in frequency of hyperhaploidy at MMII following treatment of spermatogonial stem cells with CH (Russo et al., 1984). SMN which result from stem-cell treatment may have been caused by CH's effect upon spindle microtubules during mitotic divisions in these stages, but it is unknown whether micronucleated spermatogenic cells would be capable of the many cell divisions required to progress to round spermatids. Alternatively, it is possible that a CH-induced gene mutation in some aspect of the structure or function of spindle microtubule assembly or of the synaptonemal complex could result in persistently increased frequencies of SMN containing intact chromosomes in successive cell generations. However, the mutation hypothesis is unlikely because CH is not known to be a potent mutagen.

CH also induced SMN that did not label with kinetochore antibodies or centromeric hybridization. A theoretical mechanism for the production of SMN bearing acentric fragments several cell divisions following CH treatment may involve induction of an "instability factor." This may serve to increase the probability of DNA strand breaks, alter the functioning of adjacent genes or lead to repeated chromosome "breakage-fusion-bridge" cycles, such as that described by McClintock in maize (1950). McClintock's Dissociation (Ds) locus is a

transposable element, not known to be induced by exogenous factors (Lewin, 1987) and may therefore be an unlikely model. However, transmission of induced chromosomal instability has been observed in mouse peripheral blood reticulocytes which continued to exhibit increased frequencies of MN many days following treatment with MMC or benzo[a]pyrene (Russo et al., 1993). This type of induced damage may be created by CH in the mouse male germ line as well. Further, a delay in the formation of MN from acentric fragments has been observed in plant endosperm (Bajer and Vantard, 1988), due to inability of such fragments to bind to spindle microtubules, suggesting another possible mechanism for delayed CH-induced SMN formation. At this time there is insufficient evidence to identify the mechanism of CH-induction of increased SMN frequencies several cell divisions following exposure. Studies of the intermediate stages of spermatogenesis between stem cells and early spermatids are encouraged to elucidate the nature of the cellular alterations responsible for these SMN.

Neither laboratory found evidence for a significant induction of SMN after CH treatment during early meiosis. Negative findings were also reported by Russo and Levis (1992a) after preleptotene administration of CH. However, a significant induction of SMN following preleptotene treatment with CH was previously reported by Laboratory 1 of the current study, but this finding was limited to a single dose (82.7 mg/kg; Allen et al., 1994). The authors suggested that this apparent induction of SMN by CH during DNA synthesis may indicate S-dependent clastogenic activity. A significant induction of spermatocyte hyperhaploidy has also been reported following CH treatment during preleptotene (Liang and Pacchierotti, 1988; Russo et al., 1984), though the spindle apparatus is not yet formed during this phase. The mechanism of action

of CH in these meiotic cells remains to be elucidated.

Laboratory 1 found no evidence for CH-induced SMN in mice treated in late meiosis. The literature regarding late meiotic effects is inconsistent. Studies by Russo and Levis (1992b) and Allen et al. (1994) revealed no increases in SMN at 22 or 24 hours after CH administration, but a doubling in SMN frequency at 48 hours post-treatment (Russo and Levis, 1992b), consistent with meiotic delay reported for CH (Miller and Adler, 1992). The protocol employed in the current study, in which all round spermatids are scored, may have a lower sensitivity to increases in SMN frequency than the SMN assay as it was conducted by Russo and Levis (1992b), in which only round spermatids in early steps were scored.

For somatic studies, a design employing multiple doses followed by a single sample time has been suggested to be more sensitive than the more common design that uses a single dose and multiple sample times (Arlett et al., 1989). MacGregor and colleagues (1990) demonstrated that continuous or repeated treatments of mice lead to steady-state levels of micronucleated PCEs in bone marrow and blood of mice. A multiple-dosing regimen may increase the sensitivity of the SMN assay with centromeric FISH or kinetochore immunofluorescence as well. A follow-up study employing multiple-doses followed by sampling at an interval corresponding to stem-cell administration is in progress. Additional studies will be required to characterize the persistence of the damage in the spermatogenic stem cells, a pertinent issue for the future reproductive health of the many children and young adults who are currently being treated with this agent.

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November 15th, 1994

To whom it may concern:

I, Armand C. Tcheong, fully authorize Elizabeth A.V. Nutley to use all the information and data collected during the study of spermatid micronucleus induction by the compound, chloral hydrate. She can use that information to complete her Master of Arts from the Department of Biological Sciences at San Jose State University. Thank you for your cooperation.

Sincerely,

Armand C. Tcheong

Elizabeth Nutley
825 Bay Street
Santa Cruz, CA 95060

Marek Ma
2019 Weldon Boulevard
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(313) 996-1888
November 21, 1994

Dear Ms. Nutley:

I grant you, Elizabeth V. Nutley, permission to use my work on the micronucleus project in your master's dissertation. The work was performed from 8/93 to 12/93 at Lawrence Livermore National Laboratory under the mentorship of Dr. Andrew Wyrobek.

Sincerely yours,

Marek Ma

Marek Ma

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

**HEALTH EFFECTS RESEARCH LABORATORY
RESEARCH TRIANGLE PARK
NORTH CAROLINA 27711**

November 15, 1994

Ms. Elizabeth Nutley
825 Bay Street
Santa Cruz, CA 95060

Dear Ms. Nutley:

This is to advise you that you have my permission to use the data from our collaborative study in your thesis.

Sincerely,

A handwritten signature in cursive script that reads "James W. Allen".

James W. Allen, Ph.D.
Genetic Toxicology Division

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
HEALTH EFFECTS RESEARCH LABORATORY
RESEARCH TRIANGLE PARK
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November 15, 1994

Ms. Elizabeth Nutley
825 Bay Street
Santa Cruz, CA 95060

Dear Ms. Nutley:

This is to advise you that you have my permission to use the data from our collaborative study in your thesis.

Sincerely,

A handwritten signature in cursive script that reads "Barbara W. Collins".

Barbara W. Collins
Genetic Toxicology Division