

Damaging Effects of Fourteen Chemotherapeutic Drugs on Mouse Testis Cells¹

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ABSTRACT

The harmful effects of 14 chemotherapeutic drugs on spermatogenesis in the mouse have been evaluated by studies of testicular cell killing and morphological and genetic damage produced. Male mice were given drugs as single injections at various doses up to the toxic levels. Prednisone and 6-mercaptopurine produced little or no cytotoxicity. All other drugs tested killed differentiated spermatogonia. Of these, methotrexate, cyclohexylchloroethylnitrosourea, *cis*-platinum, and mechlorethamine did not show significant stem cell killing. Bischloroethylnitrosourea, chlorambucil, 5-fluorouracil, mitomycin C, actinomycin D, and procarbazine showed some stem cell killing. Triethylenethiophosphoramide (thio-TEPA) was the only drug in this group which killed large numbers of stem cells. Only 5-fluorouracil and *cis*-platinum killed spermatocytes, and only *cis*-platinum killed spermatids. Several drugs induced chromosome breaks in treated spermatocytes. Thio-TEPA was effective in inducing chromosome translocations in treated spermatocytes and probably also in spermatocytes which originated from surviving treated stem cells.

It had been our hypothesis that the cytotoxic effects of these drugs on mouse testicular stem cells would correlate with the duration of azoospermia observed in patients. This was shown not to be the case. Thus, the cytotoxic effects of single injections of single chemotherapeutic agents on the mouse testis did not appear to be predictive of which drugs will cause long-term azoospermia in humans.

INTRODUCTION

Permanent azoospermia and sterility is now recognized as a common side effect of cancer chemotherapy in humans (43). An evaluation of which drugs are responsible for this damage would provide valuable information to the clinician. The use of drugs with lower toxicity to testicular cells may then be considered in the treatment of patients of reproductive age who have reasonable prognoses. This is very difficult to evaluate from clinical data, since combination chemotherapy is now used almost exclusively. An experimental test system is needed. Since spermatogonial stem cell survival appears, at least in the mouse, to provide a measure of the duration and permanence

of radiation- or chemotherapy-induced sterility and oligozoospermia (32), that parameter was chosen for screening the sterilizing effect of chemotherapeutic agents.

In a previous study (27), single injections of 7 chemotherapeutic drugs (ADR,⁴ bleomycin, cyclophosphamide, 1- β -D-arabinofuranosylcytosine, hydroxyurea, vinblastine, and vincristine) were given to mice. Survival levels of stem spermatogonia, differentiated spermatogonia, and other testicular cells were measured. As a next step in these studies, we have applied these same techniques to 14 additional common chemotherapeutic agents. In addition, we have also obtained some preliminary information as to the genetic toxicity of several of these agents. The rationale for this approach is that, since nearly all chemotherapeutic drugs will cause some cytotoxic and genotoxic effects on spermatogenic cells, it is only by applying the same methods to a spectrum of drugs that quantitative information on the effects of different drugs can be obtained. One goal of this study was to determine whether or not the stem cell cytotoxicity of single injections of single agents given to mice can predict the clinical results observed in humans.

Various end points could be used to assess long-term effects on fertility. Measurement of stem cell survival was considered to be most important since all differentiated cells leave the testis within 45 days. The sperm count assay for stem cell survival was chosen since it is more sensitive and rapid than fertility testing and is highly correlated with fertility (32). In general, biochemical tests have not been able to predict the effects of these drugs on sperm production and fertility, although they provide information such as mechanisms of drug action and its testicular penetration and activity. Gross histology of the testis is often of little value unless high levels of damage are observed. Quantitative germ cell counts are tedious and were not considered necessary, since striking effects of nearly all drugs were detected by sperm counts. However, semiquantitative histological analysis of each individual stage of the seminiferous epithelial cycle is more rapid and can provide a comparison of the different stages of spermatogenesis upon which these drugs act, to use as a basis for future studies of mechanisms of cytotoxicity.

A variety of methods can be used to study mutagenic effects of chemotherapy. Biochemical assays (e.g., unscheduled DNA synthesis) may provide mechanistic information but would not provide any information regarding events in stem cells. Muta-

¹ This investigation was supported by Grants CA-17364 and CA-06294, awarded by the National Cancer Institute, Department of Health, Education, and Welfare. Animals used in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Health, Education, and Welfare, NIH.

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Received April 29, 1981; accepted September 10, 1981.

⁴ The abbreviations used are: ADR, Adriamycin; LD₅₀, 50% lethal dose; MTX, methotrexate; PCB, procarbazine; BCNU, bischloroethylnitrosourea; PRED, prednisone; THIO, triethylenethiophosphoramide; CCNU, cyclohexylchloroethylnitrosourea; 5-FU, 5-fluorouracil; CDDP, *cis*-diamminedichloroplatinum II; HN2, mechlorethamine; MTC, mitomycin C; ACT, actinomycin D; DNR, daunorubicin; 6-MP, 6-mercaptopurine; CHL, bis(2-chloroethyl)aminophenylbutyric acid; MOPP, mechlorethamine-vincristine-procarbazine-prednisone combination therapy; MVPP, mechlorethamine-vinblastine-procarbazine-prednisone combination therapy.

genicity assays involving breeding and analysis of the offspring are most desirable but are expensive and time consuming. We have, therefore, chosen the spermatocyte translocation assay to measure the genotoxic effects of chemotherapeutic drugs. This assay can measure effects on stem cells and is correlated with chromosomal abnormalities in fetuses produced by breeding the treated male.

In this study, drugs were administered i.v. or i.p. Data obtained using such routes can be directly compared with clinical data. In contrast, studies which use intratesticular injection (38) cannot be compared. Administration p.o. of certain drugs might have been preferable but was not done here.

Drugs were given as single injections of single chemotherapeutic agents. This simple protocol may not be directly comparable to the clinical situation. Differences in sensitivity between slowly cycling stem cells in the unperturbed testis (37) and the regenerating stem cells that are recovering from a

cytotoxic insult may exist. Nevertheless, this study should provide baseline information necessary to compare with the effects of protracted treatments.

MATERIALS AND METHODS

Mice

C3H mice were used throughout. In most experiments, C3Hf/Kam mice maintained in our own specific pathogen-free colony were used. In some experiments, however, C3HHe/FeJ (The Jackson Laboratory, Bar Harbor, Maine), C3H/HeTex (Timco, Houston, Texas), or C3Hf/Sed (Massachusetts General Hospital, Boston, Mass.) were used. No differences between substrains were observed.

Drugs

The drugs used and their sources, preparation, and modes of injection are listed in Table 1. If the drug was injected with ethanol or Klucell

Table 1
Summary of drugs used

Drug	Other identification	Source ^a	Preparation procedure	Mode of injection
ACT	Cosmegen, dactinomycin	Merck, Sharp and Dohme, West Point, Pa.	^b	i.v.
BCNU	BICNU, carmustine	Bristol Laboratories, Syracuse, N. Y.	^b	i.p.
CCNU	Lomustine	Bristol Laboratories, ^c Syracuse, N. Y.	^d	i.p.
CHL	Leukeran, chlorambucil	Burroughs Wellcome Co., Research Triangle Park, N. C.	^e	i.p.
CDDP	cis-Platinum (NSC 119875)	Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute ^c	^f	i.p., i.v. ^g
DNR	Daunomycin (NSC 82151)	Division of Cancer Treatment, National Cancer Institute ^c	^b	i.v.
5-FU		Roche Laboratories, Nutley, N. J.	^b	i.p., i.v.
HN2	Mustargen, nitrogen mustard	Merck, Sharp and Dohme, West Point, Pa.	^b	i.p., i.v. ^h
6-MP	Purinethol (NSC 755)	Division of Cancer Treatment, National Cancer Institute ^c	^b	i.p., i.v.
MTX	Amethopterin	Lederle Laboratories, Pearl River, N. Y.	^b	i.p.
MTC	Mutamycin	Bristol Laboratories, Syracuse, N. Y.	^b	i.p.
PRED	Meticorten	Schering Corp., Kenilworth, N. J.	^b	i.p., i.m.
PCB	Matulane (NSC 77213)	Division of Cancer Treatment, National Cancer Institute ^c	^b	i.p.
THIO	Thio-TEPA	Lederle Laboratories, Pearl River, N. Y.	^b	i.p.

^a Commercial drugs in injectable form were obtained from a pharmacy unless otherwise noted.

^b Supplied as a solution or diluted with water, ethanol, or 0.9% NaCl solution according to directions on package.

^c Supplied in powdered form as a gift from listed source.

^d Dissolve 20 mg in 0.4 ml absolute ethanol. Add 0.6 ml Klucell (0.3% hydroxypropyl cellulose).

^e Dissolve 10 mg in 0.5 ml of 70% ethanol. Add 4.5 ml of 0.1 M sodium phosphate (pH 6.8). Inject quickly.

^f Dissolve in 0.1% NaCl solution and 1% mannitol.

^g Similar results were obtained with both routes of injection.

^h Similar results, except for higher LD₅₀ with i.v. injection, were obtained with both routes.

(Glogau and Co., Inc., Melrose Park, Ill.), controls were run to demonstrate that these vehicles alone had no damaging effect on spermatogenesis.

Analysis of Testicular Cell Killing

The protocol developed in a previous study (27), used here essentially with only minor modifications, is presented below.

Eleventh Day after Injection. One mouse in each dose group was killed for histological studies of testicular sections. The presence of normal numbers, markedly reduced numbers, or the absence of each cell type at each stage of the cycle of the seminiferous epithelium (36) was recorded. The stage of spermatogenesis of each cell at the time of treatment was determined. In this manner, the precise stages of differentiation of spermatogenic cells sensitive to the cytotoxic drug could be determined.

Twenty-ninth Day after Injection. Testes from at least 3 mice from each group were homogenized separately and sonicated, and sperm heads were counted. In order to facilitate more rapid processing of smaller samples, a PT7 head on the Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.) and a cup horn on the Branson sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) with a

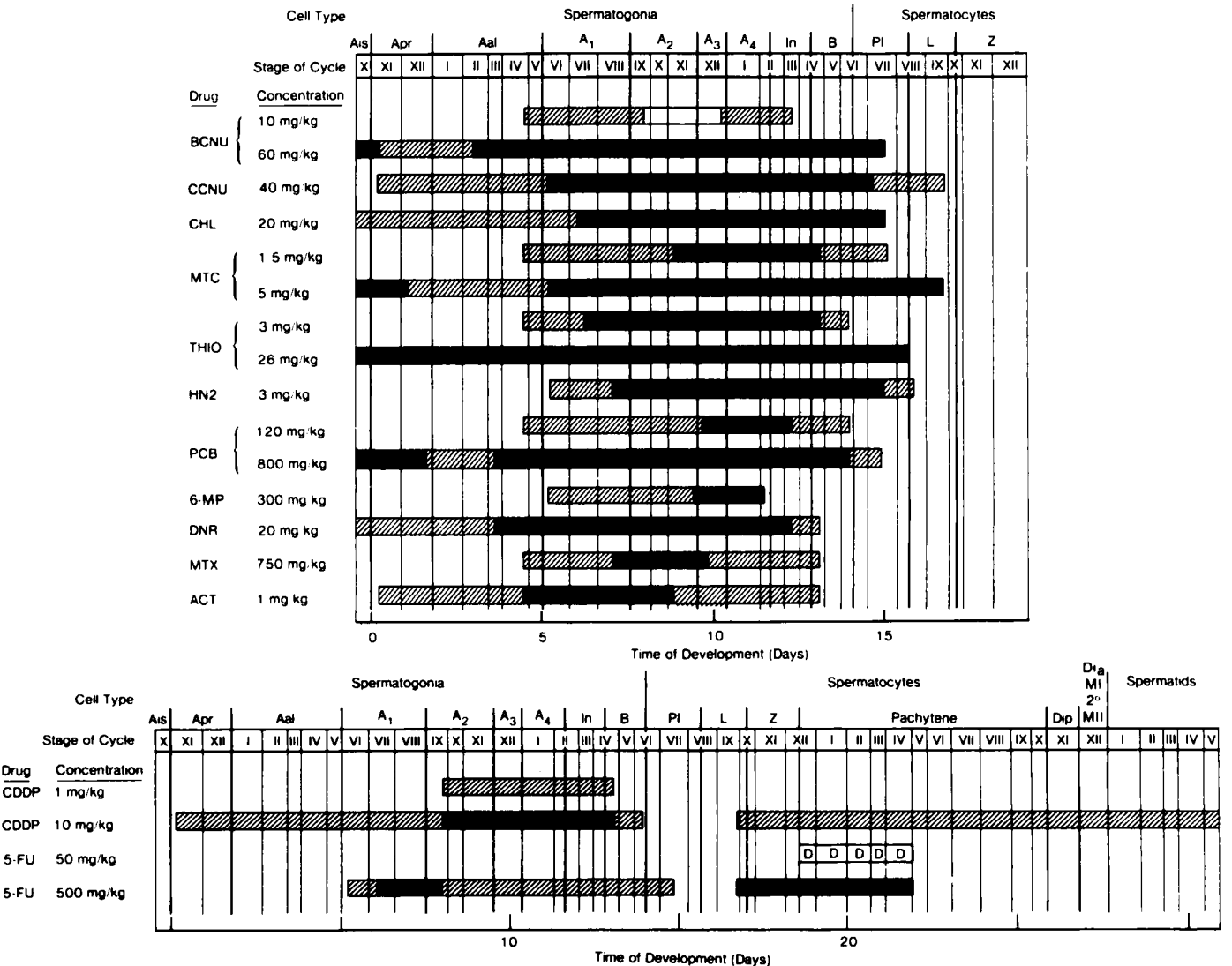
circulating cold water bath were used. The number of sperm heads per testis is a measure of the survival of the differentiated spermatogonia. The spermatogonia which differentiate into sperm heads 29 days later include the type A₁ (in Stage VIII) through the intermediate spermatogonia (at the start of Stage IV).

Thirty-fifth Day after Injection. When appreciable stem cell killing was observed by sperm head counting, testes were also prepared for histological analysis. Counts of repopulating tubular cross-sections were then performed (48). The number of spermatogenic colonies per tubular cross section (SSI) was calculated from the fraction of repopulated tubules (RI) by applying the Poisson statistical correction, $SSI = -\log_e(1 - RI)$.

Fifty-sixth Day after Injection. Sperm heads counts were obtained from at least 3 mice/dose group. The number of sperm heads on Day 56 is highly correlated with survival of testicular stem cells. As demonstrated previously, this assay may be insensitive to killing of up to 80% of the stem cells (28).

Animal Mortality

All drugs were used in concentrations up to the lethal doses. LD₅₀



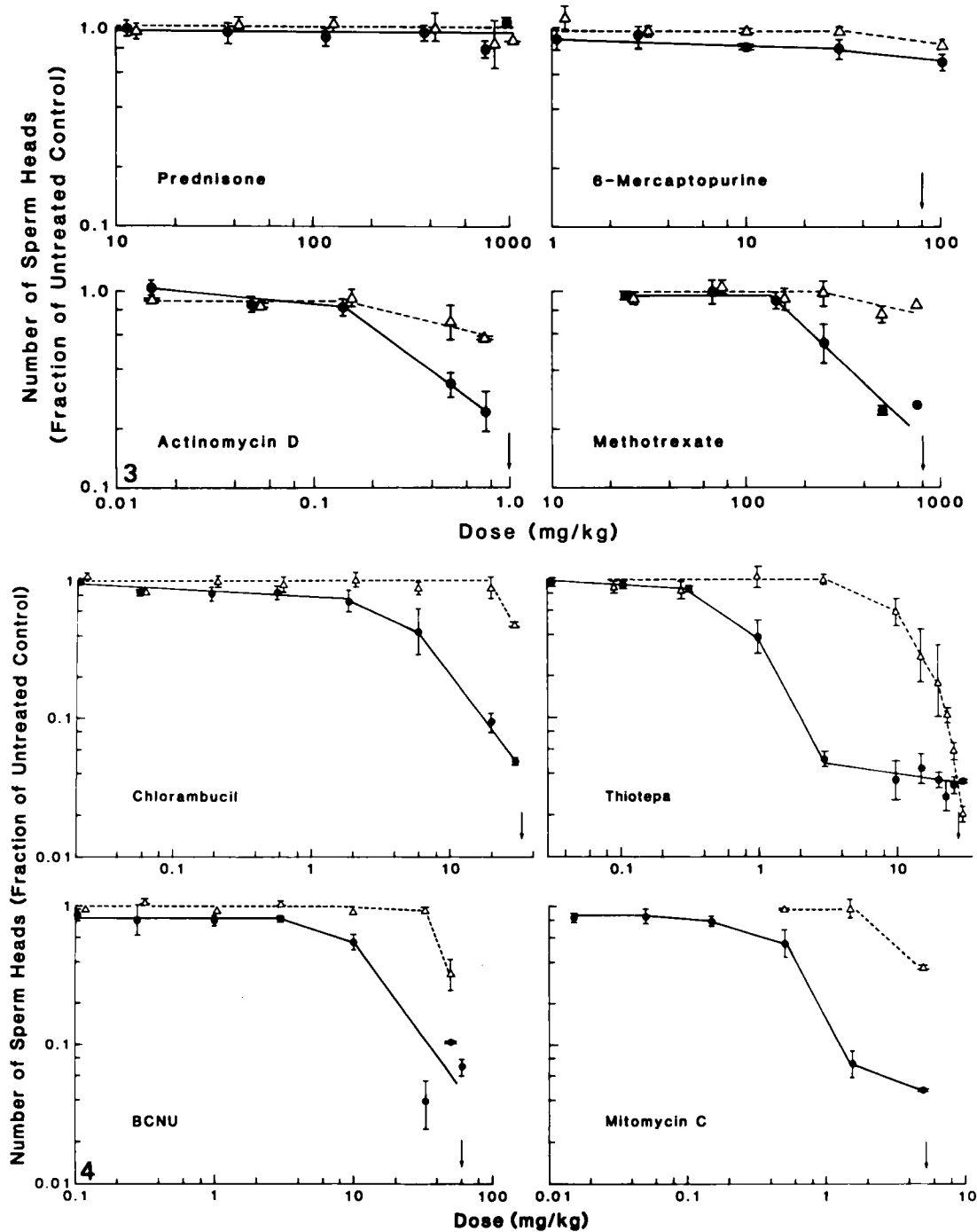
Charts 1 and 2. Specific stages of spermatogenic cells killed by various drugs as determined by analysis of histological sections 11 days after injection. □, all cells killed; ▨, some cells killed; □, no effect; □, no cells killed but many of the resulting spermids apparently diploid. A₁, A₂, and A₃ represent A-isolated, A-paired, and A-aligned spermatogonia, respectively. I_n represents intermediate spermatogonia. Pl, L, and Z represent preleptotene, leptotene, and zygotene spermatocytes, respectively.

values for animal mortality were calculated for survival at 29 days by the logit method of analysis.

Preparations for Cytogenetic Analysis

At 1, 3, 5, 11, 29, 56, and 80 days after injection, one animal from each drug group was sacrificed, and cytological preparations were made (14, 21). After removal of the tunica, the tubules were minced in 0.2 ml of balanced salt solution. The cells were suspended in a tissue culture medium, centrifuged, and then treated in 12 ml of hypotonic solution of 0.075 M KCl for 20 min at 37°. The cells were pelleted and

fixed in a Carnoy's solution. Conventional air-dried preparations were made and stained in 5% Giemsa for 10 min. Fifty cells from each mouse were scored. Some time points could not be obtained as a result of animal mortality as well as the absence of diakinesis meiotic-metaphase I cells due to spermatogonial killing. Because of the low numbers of samples in this preliminary study, data were pooled as follows. Diakinesis-meiosis I metaphase cells obtained on Days 1 to 11 were derived from cells in the spermatocyte stages at the time of treatment. Values obtained on Days 29 to 80 were derived from cells which were stem spermatogonia at the time of treatment. The signifi-



Charts 3 to 6. Relationship between testicular sperm head count/mouse (fraction of untreated control) and injected dose of various drugs displayed on log-log plots. Sperm head counts performed 29 days after injection (●) represent the number of surviving differentiated spermatogonia. Sperm head counts obtained at 56 days (Δ) represent the surviving stem cells. Points, geometric means; bars, S.E.; arrows, LD₅₀ dose for each drug.

cance in the difference between control and treated groups was determined by a χ^2 test.

RESULTS

Animal Toxicity. LD₅₀ values for animal mortality were determined (Table 2). Most deaths occurred between 4 and 15 days. Usually no general toxicity was observed at doses below the LD₅₀'s. High doses of MTX (≥ 750 mg/kg) and PCB (≥ 400

mg/kg) caused the animals to collapse after injection, but they recovered within 1 day. High doses of BCNU (≥ 33 mg/kg), PRED (≥ 800 mg/kg), and THIO (≥ 23 mg/kg) affected the motor coordination of the mice shortly after injection. Weight loss was apparent after high doses of CCNU (≥ 40 mg/kg). Cytotoxic effects on the testis cells were observed at lower doses than was animal toxicity. In addition, different drugs showed specific patterns for spermatogenic cell killing. Therefore, systemic toxicity probably has at most a minor effect on

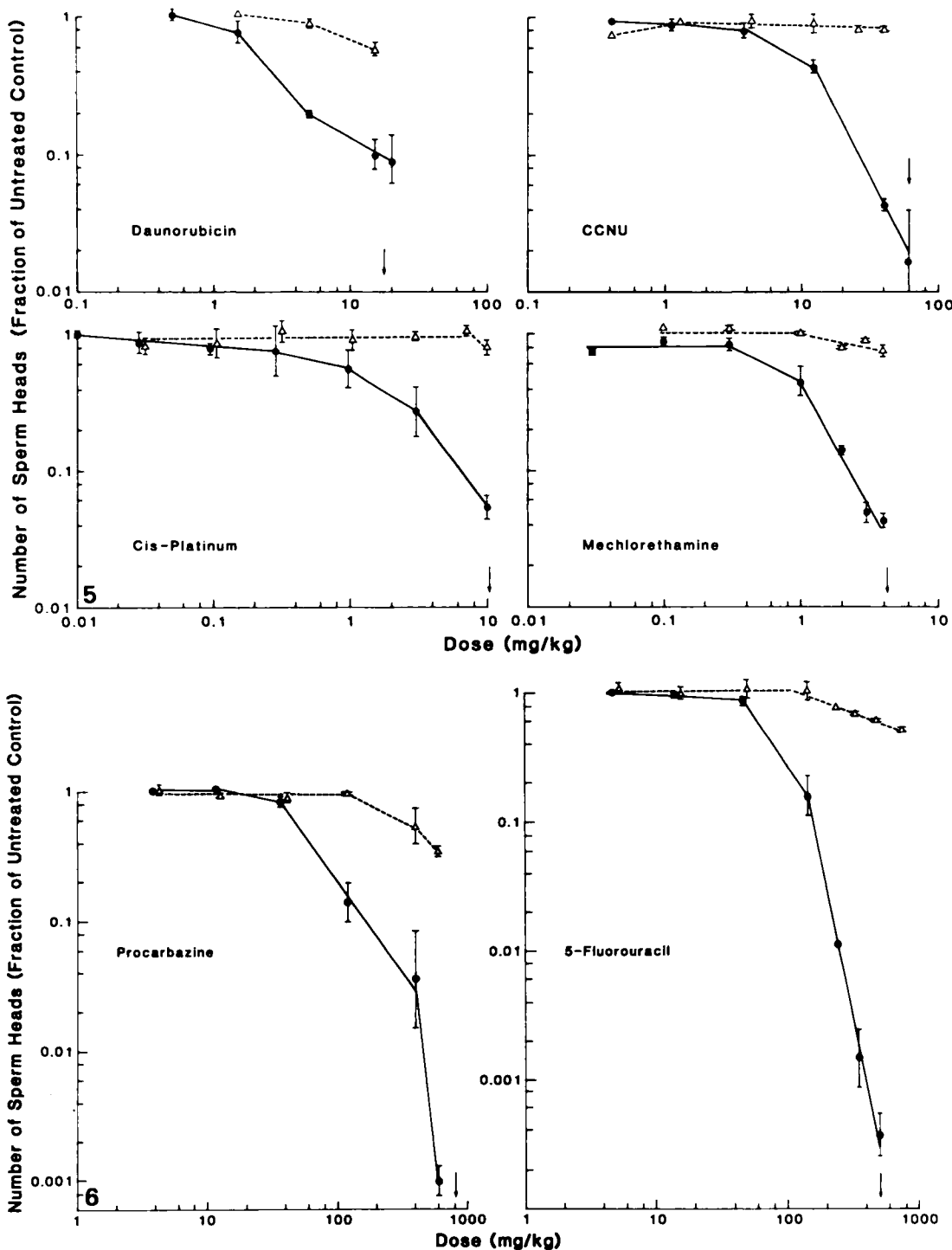


Table 2
Toxicity of acute doses of antineoplastic agents to mice and testis cells

Agent	LD ₅₀ for animal mortality (mg/kg)	LD ₅₀ for differentiated spermatogonia (mg/kg)	LD ₅₀ for stem spermatogonia (mg/kg)
ACT	1.0	0.3	≈1.3
BCNU	60	11	42
CCNU	60	9	>40
CHL	34	4	28
CDDP	11	1.1	>10
DNR	18	2.2	≈20
5-FU	530	67	750
HN2	4.4	0.8	>4
6-MP	80	>100	>100
MTX	800	260	>750
MTC	5	0.5	3
PRED	>1000 ^a	>1000	>1000
PCB	800	53	430
THIO	28	0.65	11
γ-Radiation	750 rad	53 rad ^b	320 rad ^c

^a The LD₅₀ for i.m. injection was about 600 mg/kg, but only a few animals died with i.p. injections of 800 or 1000 mg/kg.

^b From Mian *et al.* (35).

^c From Lu *et al.* (28).

killing of spermatogenic cells.

Semiquantitative Histological Analysis. Histological analysis of testes prepared from mice killed 11 days after treatment was used to obtain an overall view of drug-induced histopathology as well as to determine the specific stages at which spermatogenic cells were drug sensitive. No alterations were observed in the interstitial tissue or the structure of the tubules; the damaging effect of the drugs was primarily to the germinal cells.

The sensitivity of cells in the various spermatogenic stages was determined from histological sections (Charts 1 and 2). The general pattern of cytotoxicity observed was that differentiated spermatogonia were the most sensitive cell type, whereas the spermatocytes and spermatids were resistant. Three exceptions were found. PRED produced no detectable cell killing, even at the highest doses tested. 5-FU was the only drug which had an effect on postspermatogonial cells at doses which were insufficient to kill spermatogonia. Doses of 50 mg of 5-FU per kg affected early pachytene spermatocytes such that they produced appreciable numbers of apparently diploid round spermatids after passing through meiosis. Zygotene (but not late preleptotene or early leptotene) spermatocytes are sensitive towards high doses of 5-FU, because the damage to spermatocytes is expressed as cell killing mainly when the cells try to pass through their meiotic divisions.⁵ Eleven days after injection, the preleptotene and early leptotene cells would still be in the diplotene and diakinesis stages. CDDP, when given in a high dose, resulted in killing of some cells in all stages, from zygotene spermatocyte through Step 5 spermatid (the latest stage analyzable by this technique). Since the spermatocytes killed by CDDP were only those in the adluminal compartment (40) (behind the barrier formed by the Sertoli cells), the results could indicate that some damage to the function of the Sertoli cells occurred. With several drugs, including CDDP, HN2, MTC, and THIO, an increased number of degenerating cells in the process of meiotic division were observed.

Several patterns of differential sensitivity of the spermatogonia were observed. BCNU, MTC, PCB, HN2, and THIO preferentially killed A₃, A₄, and intermediate spermatogonia at low doses with some effect on cells up to the PI spermatocyte stage. On the other hand, 5-FU, MTX, and ACT preferentially killed A₁ and A₂ spermatogonia with the latter 2 having no effect on the preleptotene cells. At higher doses of BCNU, MTC, PCB, and THIO, killing of A_{1b} and A_{1r} spermatogonia was observed with less effect on the A_{1a} spermatogonia. The killing of A_{1b} spermatogonia by these 4 drugs is also manifest in assays for stem cell killing described below.

Several types of abnormalities in spermatids were observed as a result of drug treatments. ACT, CCNU, CDDP, DNR, 5-FU, 6-MP, MTC, and PCB produced some large round spermatids, which we presume were diploid. Other drugs, BCNU, CHL, HN2, MTX, and THIO, did not produce such cells even at the highest doses used. Another type of spermatid abnormality, observed only after CDDP, was the formation of binucleate spermatids, with nuclei fused at their acrosomes. BCNU, CCNU, PCB, and THIO caused a delay in spermiation; spermatozoa were present in Stage IX and X tubules. Several drugs resulted in the production of abnormally shaped elongated spermatids. PCB and 6-MP produced a high frequency of such cells.

Quantitative Measurement of Survival of Differentiated Spermatogonia. Testicular sperm head counts obtained 29 days after injection (Charts 3 to 6) indicated that differentiated spermatogonia were sensitive to all drugs except PRED and 6-MP. Only 2 drugs, 5-FU and PCB, could kill nearly all of these cells (10⁻³ survival). LD₅₀ values for killing of the differentiated spermatogonia by various drugs are presented in Table 2.

Quantitative Measurement of Stem Cell Survival. Stem cell survival was determined by sperm head counts 56 days after treatment (Charts 3 to 6). Several drugs, including CCNU, CDDP, HN2, 6-MP, MTX, and PRED, did produce, at the highest dose point, up to 25% declines in sperm counts, which appeared to be statistically significant. However, such small decreases could result from nonrandom variations in different groups of mice and do not demonstrate significant stem cell killing by these drugs. Seven drugs, ACT, BCNU, CHL, DNR, 5-FU, MTC, and PCB, reduced sperm head counts to between 30 and 60% of controls, indicating significant stem cell killing. Only one drug, THIO, produced strong stem cell toxicity. None of the drugs in this group produced the extent of stem cell killing observed previously with ADR (Table 3).

Stem cell killing by THIO was also demonstrated by counting nonrepopulating tubule cross-sections in histological preparations (Chart 7). Only doses above 20 mg/kg, which reduced sperm production to 5% at 56 days, were sufficient to produce an appreciable number of nonrepopulating tubules. A similar relationship between the 2 methods for assaying stem cell survival had been shown for ionizing radiation and ADR (32).

Production of Chromosomal Aberrations. Four drugs from this group (BCNU, THIO, MTC, and CDDP) were selected for study of the chromosomal aberrations produced (Table 4). BCNU and CDDP induced significant increases in the frequencies of univalents recovered at meiosis from treated spermatocytes. CDDP also increased the frequency of autosomal univalents resulting from treated stem cells. A significant increase in chromosome breakage recovered at meiosis I was also observed with treatment of spermatocytes with any of the drugs, but only BCNU-treated stem cells yielded a significantly

⁵ M. L. Meistrich, unpublished observations.

Table 3
Summary of sensitivity of spermatogenic cells to cytotoxic effects of single doses of antineoplastic agents [data from present study, Lu and Meistrich (27), and Lu et al. (28)]

Effect on stem cells	Effect on differentiating spermatogonia			
	None	Weak ($R > 0.25^a$)	Moderate ($0.1 < R < 0.2$)	Strong ($R < 0.1$)
Not significant ($S > 0.7^b$)	PRED 6-MP	MTX (>3) ^c Bleomycin (>2.5) Vinblastine (>1.5) Vincristine (>3)	CCNU (>4) CDDP (>9) ^d HN2 (>5)	1-β-D-Arabinofuranosyl- cytosine (>440) Cyclophosphamide (>7) Hydroxyurea (>50)
Some killing ($0.3 < S < 0.6$)		ACT (4)	BCNU (4) CHL (7) DNR (4) 5-FU (11) ^e MTC (6)	PCB (8)
Moderately strong killing ($S \approx 10^{-2}$)				THIO (17) γ-Radiation (6)
Very effective kill- ing ($S \approx 10^{-3}$)				ADR (3)

^a $R = LD_{50}$ for differentiated spermatogonia + LD_{50} for animal mortality.
^b $S =$ sperm head counts at 56 days at maximal dose.
^c Numbers in parentheses, LD_{50} for stem cell + LD_{50} for differentiated spermatogonia.
^d Kills spermatocytes and spermatids at high doses.
^e Early pachytene spermatocytes are extremely sensitive.

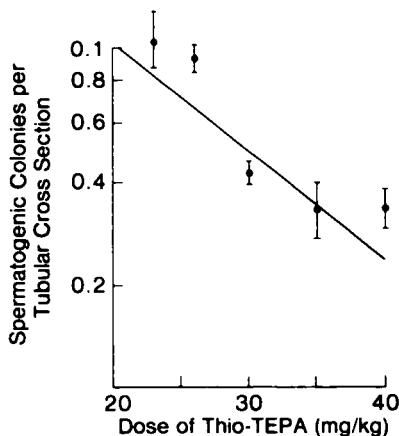


Chart 7. Spermatogenic colonies/tubular cross-section as function of dose of THIO. The number of spermatogenic colonies per tubular cross-section is proportional to the number of surviving stem cells. The line drawn is the exponential function which best fits the data by a least-squares method of analysis.

increased incidence of breaks at meiosis. THIO was the only drug which produced a significant number of multivalent translocations when spermatocytes were treated. One translocation was recovered from THIO-treated stem spermatogonia.

DISCUSSION

The results on germ cell cytotoxicity obtained here can be compared with previous studies of several drugs. Our results are consistent with the reported effects of PCB on CDF, mice (24), 101 × C3H mice (12), DBA mice (45), and rabbits (7); MTC on 101 × C3H (1, 13, 22), NMRI (30), CF₁ (16), C₃H (42), and BALB/c (19) mice; 6-MP on 101 × C3H mice (18); and 5-FU on 101 × C3H mice (22). The apparent discrepancies are listed below. With 101 × C3H mice, 6-MP showed a much higher (about 270 mg/kg) LD₅₀ for animal mortality (18). Measurements of the ability of sperm to fertilize ova indicated that PCB- (12) or MTC- (13, 22) treated spermatocytes were incapable of producing fertile sperm. Thus, the lack of cell killing

Table 4
Percentage of cells with chromosome damage in diakinesis-meiosis I figures derived from cells treated in prior stages

Agent	Dose (mg/kg)	Cell stage treated									
		Spermatocytes ^a					Stem cells ^b				
		Cells scored	XY univalents	Auto-somal univalents	Breaks	Translocations	Cells scored	XY univalents	Auto-somal univalents	Breaks	Translocations
Control	0	400	15	5	0	0	400	15	5	0	0
BCNU	33	200	22 ^c	9	4 ^d	0	100	22	3	2 ^c	0
THIO	20	150	10	9	3 ^e	3 ^c	100	13	8	1	1
MTC	5	200	18	4	2 ^c	0.5	50	10	10	0	0
CDDP	10	200	24 ^c	9 ^c	4 ^d	0	50	32 ^e	4	2	0

^a Abnormalities observed in diakinesis-meiosis I metaphase were derived from spermatocytes treated between 1 and 11 days before sacrifice.
^b Abnormalities observed were derived from stem cells treated at least 26 days before sacrifice.
^c Significantly different from control at $P \leq 0.05$.
^d Significantly different from control at $P \leq 0.01$.
^e Significantly different from control at $P \leq 0.001$.

does not mean that fertile sperm must be produced. The doses required to reduce [^3H]thymidine incorporation in rat testes 1 day later by 50% (23) differ from our results. Comparing doses on a mg/sq m basis (17), our LD₅₀ values for killing differentiated mouse spermatogonia by cyclophosphamide, CCNU, CHL, THIO, and PCB are 5- to 10-fold lower than the results of the DNA synthesis inhibition assay. The discrepancy may be a result of the different stages of spermatogonia being assayed, rather than a species difference. In general, our results are comparable to data obtained with other strains of mice and with rabbits.

Only damage to the germinal cells was detected in this study. Leydig cells, in mice treated with chemotherapeutic agents, appeared normal. Previously (33), we had shown that Leydig cell atrophy can be recognized under conditions which markedly suppress leuteinizing hormone levels. Furthermore, chemotherapy did not produce clinically significant damage in nongerminal cells of the testis, pituitary, or hypothalamus (8, 43). Presumably, testosterone synthesis by Leydig cells is still active after drug treatment, but this should be checked in future studies.

It is important to know if the survival of stem spermatogonia, spermatocytes, and spermatids indicates resistance of these cells or lack of drug penetration into the seminiferous tubules. All drugs tested, with the exception of PRED, killed differentiated spermatogonia, which are in the same compartment of the tubules as the stem cells. Thus, drug penetration to the stem cell is not a limitation. The spermatocytes and spermatids, on the other hand, are separated from the spermatogonia by Sertoli cell junctions (40). However, data on chromosomal aberrations, mutagenesis, ultrastructural alterations, and binding of radioactive drugs demonstrate that BCNU, CDDP, PCB (12), MTC (13), ACT (5), THIO (38), and HN2 (38) can penetrate the blood-testis barrier to reach spermatocytes and spermatids. Thus, the resistance of these cells cannot be ascribed to a complete lack of drug penetration. A more likely explanation for the greater sensitivity of differentiated spermatogonia is that most chemotherapeutic drugs preferentially kill actively cycling cells (11).

The cytotoxic effectiveness of different drugs towards the differentiated and stem spermatogonia were compared in 2 ways. First, the ratio of LD₅₀ doses for differentiated spermatogonia to those for animal mortality (*R*) and the survival of stem cells at the maximal dose (*S*) were used to classify the various agents (Table 3). There is limited correlation between this classification of drugs and their mechanisms of action. The *Vinca* alkaloids, which are mitotic poisons, are not toxic to stem cells, presumably because these cells are very slowly cycling (37). Similarly, drugs that affect the availability of DNA precursors (6-MP, MTX, 1- β -D-arabinofuranosylcytosine, and hydroxyurea) are also nontoxic to stem cells. The one exception is 5-FU, which also acts by interfering with RNA synthesis. Another explanation for the effectiveness of 5-FU is that its active metabolite has a much longer intracellular half-life (7 to 9 days) than, for example, that of 1- β -D-arabinofuranosylcytosine (34, 41). The stem cells may be triggered into cycle by 5-FU while the drug is still active. All alkylating agents (CCNU, HN2, BCNU, CHL, CDDP, cyclophosphamide, MTC, and THIO) showed strong killing of differentiated spermatogonia but varying degrees of stem cell killing. A second method for classifying agents was by the ratio of LD₅₀ for stem cell killing to that for

differentiated spermatogonia killing (Table 3). This ratio is greater than one in all cases, indicating the greater sensitivity of the rapidly dividing spermatogonia, than of the stem cells. The ratio is generally low for intercalating antibiotics (ADR, ACT, and DNR), higher for alkylating agents, and the highest for antimetabolites (1- β -D-arabinofuranosylcytosine, hydroxyurea, and 5-FU). The high ratio in the last category is expected since these drugs primarily affect DNA synthesis and should be more specifically cytotoxic for differentiated spermatogonia.

Next the question of whether or not data on cytotoxic effects observed in the mouse can be applied to humans should be considered. Table 5 represents an attempt to compare the experimental results with clinical data. The most cytotoxic drug, among those tested, towards murine stem cells, was ADR. In contrast, this drug did not produce permanent sterility in humans. On the other hand, combination chemotherapy with MOPP or MVPP produced permanent azoospermia in most patients. Of these agents, only PCB and perhaps HN2 were even slightly cytotoxic to mouse testicular stem cells. We estimate that 6 courses of MOPP or 8 courses of MVPP therapy will reduce mouse testicular stem cell survival to 1% or 0.03%, respectively. Recovery of sperm production in the mouse occurred after these levels of stem cell killing (32). It is impossible to compare the experimental and clinical data in the case of cyclophosphamide, since sufficient dose could not be administered in a single injection to produce stem cell killing. In the case of CHL, a crude calculation of the dose necessary to reduce stem cell survival to 10⁻⁵ does provide a good estimate of the dose at which permanent azoospermia is observed in humans.

There are several possible explanations for the discrepancies between the experimental and clinical data. (a) The response of the regenerating stem cell may differ from that of the stem cell in steady state; single injections of drug may not yield the same results as multiple injections. (b) Synergistic and antagonistic effects between drugs may occur during combination chemotherapy; the use of single drugs may not be representative. (c) The action of these drugs in humans may differ markedly from that in the mouse. In the case of ADR, the third possibility is most likely. An interspecies comparison of the pharmacokinetics, stem cell killing, and duration of azoospermia produced by this drug should be performed to understand the causes of this discrepancy. With cyclophosphamide and CHL, protracted administration over several months is necessary to obtain appropriate experimental doses to properly compare with the clinical data. In the case of MOPP or MVPP, drugs should be given to mice according to their clinical schedule, first singly and then in combination. In addition, experiments must be designed to understand the fundamental reasons for differences in sensitivity of testicular stem cells to single and fractionated treatment.

The cytogenetic effects of anticancer drugs can also have serious consequences. Translocations could result in heritable mutations. Breaks could cause embryonic lethality. The genetic consequences of univalency are unknown, but it is possible that it could result in nondisjunction. In general, greater damage is induced by chemicals when cells are treated as spermatocytes than as stem cells (2, 6, 26). Treatment of stem cells with CDDP, nevertheless, did significantly increase univalency. Previous studies had indicated that BCNU (47) but not MTC (1) treatment of stem cells could increase univalency. Several

Table 5
Comparison of experimental data with clinical results in adult patients

Regimen/no. of courses	Drug	Total dose/course (mg/sq m) ^a	Equivalent dose to mouse/course ^b (mg/kg)	Stem cell survival/course ^c	Calculated stem cell survival ^d	Patients with sperm at ≥1 yr/total patients
MVPP/8 (3, 8) ^e	HN2	12	3.6	0.76	2.9×10^{-4}	1/8 4/64
	Vinblastine	12	3.6	0.95		
	PCB	1,400	424	0.50		
	PRED	350	106	1.0		
	Combination			0.36 ^f		
MOPP/6 (46, 49)	HN2	12	3.6	0.76	1.1×10^{-2}	1/8
	Vincristine	2.8	0.8	1.0		
	PCB	1,000	303	0.62		
	PRED	550	166	1.0		
	Combination			0.47		
ADR + cyclophosphamide/9 + MTX/6 (44)	ADR	60	18	<10 ⁻⁴	0	9/12
	Cyclophosphamide	600	180	1.0		
	MTX	50 → 250	15 → 76	1.0		
	Combination			<10 ⁻⁴		
ADR/7 + various drugs (10)	ADR	50	15	<10 ⁻⁴	0	6/8
Cyclophosphamide/1 ^g (15, 20, 25, 39)	Cyclophosphamide	>12,000	>36,000	1.0 at 200 mg/kg	1.0	1/10
		<12,000	<36,000			6/7
Chlorambucil/1 ^g (9, 31)	CHL	228-1,440	70-440	0.48 at 30 mg/kg	0.2 to 2×10^{-5h}	5/5
		1,830	560		10^{-6h}	0/1

^a When data were given in mg, an average body surface area of 1.8 sq m was assumed.

^b Human dosages were extrapolated to mouse on a mg/sq m basis (17) and then converted to mg/kg by dividing by 3.3, which is the ratio of weight to surface area for a 26-g mouse.

^c Calculated from testis sperm production at 56 days after treatment.

^d The survival for each course was multiplied by itself the number of times the course was repeated.

^e Numbers in parentheses, references.

^f The stem cell survival after the combination was assumed to be the product of survivals of each individual drug.

^g Cyclophosphamide and CHL were given daily over a period of several months and not divided into courses.

^h Calculated by taking the survival at 30 mg/kg (0.48) and raising it to a power equal to the dose + 30 mg/kg.

agents appeared to increase the frequency of breaks recovered in spermatocytes after treatment of stem cells, as observed previously with ADR (4). In addition, the observation of a translocation induced in stem cells by THIO indicates that the genetic damage to stem cells may be expressed in the resulting spermatocytes, as was the case of ADR. A previous study (29) had also indicated induction of translocations in spermatogonia by THIO. Thus, the 2 agents that were most cytotoxic towards stem cells were also most genotoxic. These results demonstrate the importance of pursuing further study of the mutagenicity of chemotherapeutic agents on germ cells.

In conclusion, this study has characterized the differences in sensitivity of specific spermatogenic cell types to 14 chemotherapeutic agents. Quantitative measurements of stem cell survival represent a first step in an attempt to develop an experimental system to test the sterilizing effects of chemotherapeutic drugs.

ACKNOWLEDGMENTS

We thank Debbie Palmatary and her staff for the supply and care of the mice used in this study, Betty Reid and Cheryl Collie-Bruyere for technical assistance, Ann McCarver for preparation of the manuscript, and Dr. T. C. Hsu for guidance in the cytogenetic studies.

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