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Preconception Urethane or Chromium(III) Treatment of Male Mice: Multiple Neoplastic and Non-neoplastic Changes in Offspring

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Increase in neoplasia in offspring after preconception exposure of parents presents puzzling features such as high frequency of effects and lack of Mendelian inheritance. The present study examined the hypothesis that preconception carcinogenesis involves an increase in the rate of occurrence of neoplasms with a spontaneous incidence. Male NIH Swiss mice (12 per group) were exposed 2 weeks before mating (once, ip) to urethane (1.5 g/kg) or chromium(III) chloride (1 mmol/kg). Offspring (48–78/sex/group) were examined for all grossly apparent changes when moribund or at natural death, followed by histopathological diagnosis and statistical analysis. Significant exposure-related changes occurred in multiple organs. Ten to 20 percent of offspring showed changes related to paternal exposure, including at least one sired by most treated males. Pheochromocytomas occurred in both male and female offspring after both treatments, with none in controls. These neoplasms are rare in mice and suggest endocrine dysfunction as a component of preconception carcinogenesis. This was supported by increases in thyroid follicular cell and Harderian gland tumors, ovarian cysts, and uterine abnormalities. Lung tumors were increased in female offspring only. Effects seen in offspring only after paternal urethane exposure were an increase in preneoplasia/neoplasia in the glandular stomach (males) and in females, increased lymphoma but decreased incidence of histiocytic sarcoma. Increases in incidence of male reproductive gland tumors and of renal non-neoplastic lesions occurred only after chromium exposure. Thus, preconception exposure of fathers to toxicants had a significant impact on both neoplastic and non-neoplastic changes in almost all tissues in which these lesions often occur naturally during the aging process. © 1999 Academic Press

Numerous epidemiological studies have suggested significant correlations between paternal exposure to chemical carcinogens or radiation and incidence of childhood cancers, including brain tumors, Wilms' tumors of kidney, leukemias, hepatoblastomas, retinoblastomas, and sarcomas in soft tissues (Savitz and Chen, 1990; Tomatis *et al*., 1992; Yamasaki *et al*., 1992; Bunin *et al*., 1992; Tomatis, 1994; Olsen *et al*., 1991; Bhatia and Neglia, 1995). This association has been particularly frequent and strong for parental occupational exposures to metals (Bunin *et al*., 1992; Tomatis, 1994; Olsen *et al*., 1991). An especially important and controversial issue has been a possible increase in childhood leukemia as a result of paternal exposure to radiation (Gardner, 1993; Shu *et al*., 1994; Satoh *et al*., 1996; Draper *et al*., 1997; Little *et al*., 1995). Although the latter risk appears to be discounted in current opinion (Satoh *et al*., 1996; Draper *et al*., 1997; Little *et al*., 1995; Tawn, 1995; Wakeford and Parker, 1996), definitive evaluation of such risks could be greatly facilitated by discovery of the underlying molecular mechanisms of preconception carcinogenesis.

One approach to this discovery is use of animal models of preconception carcinogenesis. A convincing though largely descriptive literature demonstrates that exposures of male rodents, *in utero* or as adults in the weeks before mating, to an assortment of chemical carcinogens (urethane, *N*-ethylnitrosourea, *N*-nitrosodiethylamine, 4-nitroquinoline *N*-oxide, diethylstilbestrol, or cyclophosphamide), or to X- or neutron irradiation can result in significant increase in the incidence of tumors in their progeny and sometimes later generations (for reviews see Tomatis *et al*., 1992; Yamasaki *et al*., 1992; Tomatis, 1994; Mohr *et al*., 1995; Watanabe *et al*., 1996; Lee *et al*., 1998; Lord *et al*., 1998; Daher *et al*., 1998). Target organs include the nervous system and uterus of rats; lung, lymphoid tissue, ovary, uterus, liver, intestine, and skin of mice; and neuroendocrine cells, forestomach, and uterus of hamsters. Many of these experiments have been carried out only once or with only one type of animal model. Efforts to reproduce critical findings have sometimes been unsuccessful, as for causation of nervous system tumors by paternal exposure to *N*-ethylnitrosourea in rats (Tomatis *et al*., 1990) and of lung

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tumors in mice by paternal X-ray treatment (Cattanach *et al*., 1995, 1998). Again, understanding of this confusing situation would be helped by information as to mechanism.

A number of the characteristics of transgenerational carcinogenesis, as revealed by the published literature, have suggested that the mechanism is not structural gene mutation (Anderson *et al*., 1994). These include the high incidence of effects and lack of Mendelian inheritance with some animal models. In addition, there is a considerable body of data, seldom referenced in the carcinogenesis literature, showing transgenerational effects of parental treatments on physiological end points in offspring, which can reach 100% in incidence (Campbell and Perkins, 1988). On the basis of these observations, we have hypothesized that the mechanism of preconception carcinogenesis involves epigenetic changes resulting in altered gene expression in the offspring, perhaps as a result of effects on gene imprinting (Anderson *et al*., 1994). This hypothesis would explain most of the observed characteristics of preconception toxicity, including lack of reproducibility, since alterations in gene expression would be more dependent on the specific strain of animal, laboratory conditions, etc., than would gene mutation.

A corollary of this hypothesis is that the neoplasms that would be most affected would be those with significant spontaneous occurrence in the species and strain of animal. Thus, the neoplasms would be initiated by inherited or spontaneous genetic changes and then stimulated in their expression or growth by the changes in gene expression effected by preconception exposure. Indeed, the tumors most often increased in these experiments are those also seen in controls, including those of lung and liver and hematopoietic and female reproductive tissues in mice (Nomura, 1989), mammary gland and pituitary in rats (Tomatis, 1979), and uterine, forestomach, and neuroendocrine tumors in hamsters (Mohr *et al*., 1995). Tumors with low spontaneous incidence but ease of induction and promotability, e.g., skin tumors of mice, may also be prone to increase.

Most of the spontaneous neoplasms in rodents, as in man, occur during old age, yet few preconception carcinogenesis studies in mice have followed offspring systematically through old age until natural death, and few have included quantification of non-neoplastic changes, that might be expected, from the above hypothesis, to result from altered gene expression.

In the present study, we have utilized two chemical carcinogens: urethane, a preconception carcinogen in mice leading to increased lung tumors in offspring (Nomura, 1978, 1982), and chromium(III). Chromium(III) is a constituent of welding fumes and was found to be a preconception carcinogen for lung and possibly lymphoid tissues in our preliminary study with mice (Anderson *et al.*, 1994). After exposure of Swiss male mice to these agents in the current study, most of their offspring were allowed to live until natural death, and all grossly detected pathological changes were systematically examined and quantified. The results show that paternal exposure to both agents caused numerous significant changes in incidence of aging-associated neoplastic and non-neoplastic abnormalities, including both increases and decreases. The results overall are consistent with a mechanism involving alterations in gene expression. In addition, pheochromocytomas, a rare neoplasm in mice, occurred in offspring as a result of paternal treatment.

METHODS

Swiss Cr:NIH(S) mice were obtained from the Animal Production Area of the Frederick Cancer Research and Development Center and maintained under standardized pathogen-free conditions. Male mice, 6 weeks old, were treated once ip with 1 mmol/kg of freshly prepared $CrCl_3 \cdot 6H_2O$ (Aldrich Chemical Co., Milwaukee, WI) in sterile distilled water adjusted to pH 4.1 with NaOH, or 1.5 g/kg urethane (Sigma Chemical Co., St. Louis, MO) in sterile distilled water. These doses had been shown to have no major toxic effects on the internal organs of the male or on their reproductive performances. Controls received a similar administration of water. Two weeks later, each male was offered five 8-week-old females. This timing ensured that sperm utilized in fertilization would have been exposed postmeiotically, a stage of high sensitivity to preconception carcinogenic effects (Nomura, 1978). The females were held for an additional 3 weeks and housed separately when visibly pregnant. Offspring were weaned at 4 weeks. Female offspring were held without further treatment until natural death or killed when moribund. A subset of the male offspring were killed at approximately 12 and 15 months, to assess the utility of these end points in time. The remainder were held until natural death or morbidity. Group compositions are given in Table 1.

All mice were subjected to complete necropsy, and tissues were fixed in formalin. Lungs were inspected for tumors with a magnifying glass, a procedure that has been found by us and by others to permit quantification of tumors of the peripheral lung with at least 90% accuracy. Only tumors that were at least 1 mm in largest dimension were included in the analysis. At least 10% of these tumors were examined and confirmed histologically in each group. Representative lungs with tumors and all of those cases for which the presence of other neoplasms suggested the possibility of pulmonary metastases were examined histologically. All other masses and lesions were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for pathological diagnosis.

For statistical analysis, comparisons among the survival curves were made using the Cox proportional hazards methodology as employed in the computer program developed and described by Thomas *et al*. (1977). Comparisons among tumor incidences were made by the Fisher exact test (FET) or the χ^2 test of homogeneity, depending upon the number of proportions being compared. Tumor multiplicities were compared by the nonparametric Kruskal–Wallis and Wilcoxon rank-sum (Mann–Whitney) methods (Hollander and Wolfe, 1973). Tests of the homogeneity of the proportions of tumor bearers per litter and per sire for many of the tissues/organs involved were performed by use of the correlated binomial C(α) test statistic as described by Tarone (1979).

Since the hypothesis to be tested was that preconception exposures would increase disease incidence in the progeny, one-tailed tests for such changes are appropriate. However, two-tailed tests are regarded as more stringent. A further aspect of the hypothesis was that changes could be small in magnitude, compared to controls. Therefore, we have stated both one- and two-tailed values for many comparisons and have included p values of ≤ 0.10. We regard differences where $p \leq 0.05$ by two-tailed test as most convincing; differences in the direction of the hypothesis where $p \leq 0.05$ by one-tailed test as probably real; and differences where $p \leq 0.10$ by one-tailed test as suggestive. For pairwise tests where controls are compared with the two treatment groups, the actual p values for the individual comparisons are reported; if experiment-wise probabilities are desired in these cases, the reported p values would be multiplied by 2, according to the Bonferroni correction (Miller, 1966). We have not formally applied the Bonferroni correction, since the use of multiple

TABLE 1
Reproductive Success and Numbers of Mice Studied

Group, paternal treatment	Percent females impregnated ±SD	No. of fathers with surviving offspring	Total no. of litters	Av. no. born per litter ±SD	No. litters weaned	Av. no. weaned ±SD	No. male offspring killed at 12 mo[b]	No. male offspring killed at 15 mo[b]	No. offspring for lifetime study[a]	
									Female	Male
1 Urethane	66 ± 31	12	43	7.6 ± 1.9	35	5.9 ± 3.3	None	20	78 (20)[c]	54 (20)
2 $CrCl_3$	73 ± 23	11	44	8.4 ± 1.9	36	6.3 ± 3.4	20	None	71 (20)	72 (21)
3 Control	71 ± 28	11	44	8.5 ± 2.3	36	6.4 ± 2.4	20	18	71 (23)	48 (14)

Note. Each male was bred with 4–6 females. All of the offspring in 11 or 12 litters from each group were killed at 6 weeks of age for measurement of biochemical and molecular changes (data not shown).

[a] All mice dying naturally or killed when moribund.

[b] Mice used for serial sacrifice.

[c] Number in parentheses is number of litters represented.

comparison procedures for complex data sets is controversial (Bailar and Mosteller, 1992).

Levels of 8-hydroxydeoxyguanosine (8-oxo-dG) were measured as described by Adachi *et al.* (1995).

RESULTS

Reproductive success and offspring survival. Fertility and fecundity records and the numbers of offspring utilized for lifetime study are given in Table 1. One chromium-treated and one control father did not produce viable offspring. No significant differences were observed in the average percentage of females impregnated among those mated with treated or with control males. There were no major effects on numbers of offspring born or surviving to weaning. At 6 weeks of age, offspring from 11 or 12 litters per group were killed, and tissues were frozen for later biochemical and molecular analysis (data not shown). Two of the urethane fathers were not represented among the offspring analyzed at natural death.

Additional groups of male offspring from several litters per group were killed at 12 and 15 months in order to assess the usefulness of these end points in time for detection of increases in lung tumor incidence. The numbers of lung tumors discovered were 3/20 from chromium-treated fathers and 1/20 from control fathers at 12 months, and 7/20 from urethane-treated fathers and 6/18 from control fathers at 15 months. In view of these inconclusive or negative data, no additional interim kills were planned. All remaining mice were examined when moribund or at natural death. Two Harderian gland tumors were discovered in the chromium treatment group at 12 months, and non-neoplastic abnormalities were found in the reproductive tract, liver, and kidney at both time points. Therefore, the data for these tissues from the interim kill mice were included in the statistical analyses (see Tables 2, 7, and 8).

Survival curves for each sex were similar for each group, with males from urethane-treated fathers surviving a slightly shorter time on average compared with males from the other groups (Fig. 1). The percentages surviving at 900 days of age were 25.5%, 33.5% and 30% for urethane-, chromium-treated, and control groups, respectively. The average ages at natural death (days ± SD) were 669 ± 211 for the urethane-group males, compared to 743 ± 197 for the chromium-group males and 730 ± 203 for control males, differences not quite of statistical significance. The average ages at death for female offspring were quite similar, 856 ± 124, 848 ± 128, and 857 ± 160 days for Groups 1, 2, and 3, respectively.

Adrenal, thyroid, Harderian gland, and glandular stomach lesions and incidental neoplasms. One of the most novel findings of the study was the occurrence of adrenal pheochromocytomas in offspring of both sexes and chemical treatment groups, plus one adrenal cortical adenoma (Table 2). No such tumors occurred in control mice. Although the incidences of these tumors were low, they were of statistical significance. Non-neoplastic changes in adrenal tissue were also found and appeared to be treatment-related in the female mice (Table 2 and legend); these had no obvious correlation with the occurrence of pheochromocytoma.

In the thyroid gland, follicular adenomas and carcinomas

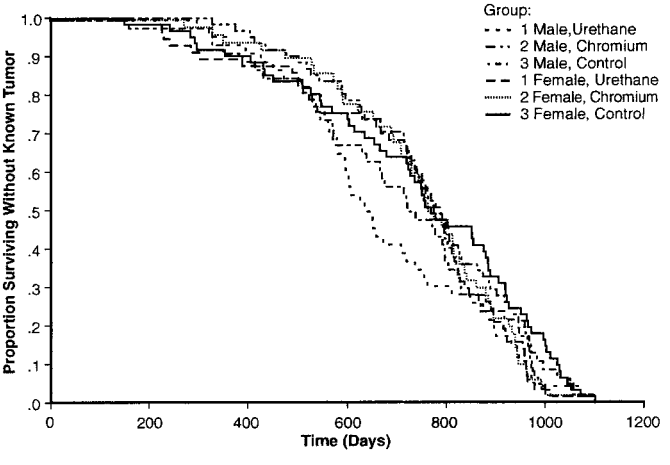

FIG. 1. Kaplan–Meier survival curves for each group and sex.

TABLE 2
Endocrine, Harderian Gland, and Gastric Lesions and Incidental Tumors in Offspring of Treated and Control Males

Organ	Female offspring			Male offspring		
	Group 1, urethane ($N = 78$)	Group 2, $CrCl_3$ ($N = 71$)	Group 3, control ($N = 71$)	Group 1, urethane ($N = 54$)	Group 2, $CrCl_3$ ($N = 72$)	Group 3, control ($N = 48$)
Adrenal gland						
Tumors	3 (4)[a]	5 (7)[b]	0[a, b]	3, incl. 1 cortical adenoma (6)[a]	2 (3)[b]	0[a, b]
Any lesion	12 (15)	13 (18)[c]	5 (7)[c]	4 (7)	4 (6)	4 (8)
Thyroid gland						
Tumors	5 AD (6)	5 AD (7)[d]	2 AD (3)[d]	1 AD (2)	5 (3 AD, 2 CA) (7)[d]	0[d]
Any lesion	8 (10)	7 (10)[e]	3 (4)[e]	1 (2)	8 (11)[e]	2 (4)[e]
Harderian gland	7 (5 AD, 2 CA) (9)	4 AD (6)[f]	3 (1 AD, 2 CA) (4)[f]	8 AD (11) ($N = 74$)	14 AD (15)[f] ($N = 92$)	5 (4 AD, 1 CASRC) (6)[f] ($N = 86$)
Glandular stomach	0	(1 squamous cell papilloma, nonglandular)	1 CA (1)	10 (3 AD, 3 CA, 4 atypical hyperplasia) (19)[g]	1 AD (1)[g]	2 AD (4)[g]
Pituitary, pars distalis	4 (3 AD, 1 CA) (5)	2 AD (3)	4 (3 AD, 1 CA) (6)	0	0	0
Other tumors	2 squamous cell CA (oral cavity)	3 trichoepitheliomas; 1 choroid plexus tumor	3 squamous cell CA (2 oral cavity, 1 head); 1 squamous papilloma, skin	0	1 sc CA; 1 oligodendroglioma; 1 pancreatic islet cell AD	1 squamous cell CA (oral cavity); 1 transition cell CA of ureter

Note. Values in parentheses are percentages. AD, adenoma; CA, carcinoma; CASRC, carcinosarcoma.

[a] All tumors were pheochromocytomas, except for the cortical adenoma noted. For all adrenal tumors, females plus males vs controls: $p = 0.031$, two-tailed FET. For pheochromocytomas, females plus males, $p = 0.062$ and 0.039, two- and one-tailed FET, respectively.

[b] Females vs controls: $p = 0.058$ and 0.029, two- and one-tailed FET, respectively; females plus males vs controls: $p = 0.017$ and $= 0.013$, two- and one-tailed FET, respectively.

[c] Included lipogenic pigment cells, subcapsular cell hyperplasia, hyperplasia or cytoplasmic vacuolation of the zona fasciculata, cortical atrophy, or medullary hyperplasia. Group 2 vs Group 3, $p = 0.075$ and 0.038, two- and one-tailed FET, respectively.

[d] All tumors were follicular. Males vs controls: $p = 0.073$, one-tailed and 0.082, two-tailed FET; males plus females vs controls: $p = 0.071$ and $= 0.036$, two- and one-tailed FET, respectively.

[e] Included dilated follicles, follicle cell hyperplasia. Males plus females vs controls: $p = 0.064$ and $= 0.045$, two- and one-tailed FET, respectively.

[f] All males in the study included, since two adenomas were found in scheduled-kill mice of Group 2, one at 12 months and one at 15 months. Males vs controls: $p = 0.052$ and $= 0.035$, two- and one-tailed FET, respectively; males plus females vs controls: $p = 0.065$ and $= 0.040$, two- and one-tailed FET, respectively.

[g] Group 1 vs Group 3, all preneoplastic and neoplastic lesions, $p = 0.032$ and 0.024, two- and one-tailed FET, respectively. Group 1 vs Group 2, adenomas/carcinomas only: $p = 0.024$, one-tailed, and 0.043, two-tailed FET; all preneoplastic and neoplastic: $p = 0.0009$, two-tailed FET.

occurred in offspring of both sexes of both treatment groups, with fewer in control females and none in control males (Table 2). The effect was most prominent in the chromium group. Non-neoplastic changes, including dilated follicles and follicular cell hyperplasia, also showed a possible increase in this group, but did not correlate clearly with tumors. Incidental changes included cyst formation, lymphocyte infiltration, and congestion (data not shown).

Harderian gland adenomas were more common in the treatment-group offspring of both sexes, compared with controls; the largest difference was a 2.5-fold increase in these tumors in the male offspring of the chromium group (Table 2). Hyperplasia, cystic degeneration, and chronic active inflammation were the only incidental non-neoplastic changes observed in this tissue.

In the glandular stomach, adenomas and carcinomas occurred mainly in the male offspring of the urethane group (Table 2). Severe atypical hyperplasias also were observed, and since these lesions are considered to be precursive to neoplasia (Odashima, 1979), they were combined with neoplasms for statistical analysis ($p = 0.03$ vs controls). One case of gastric erosion also occurred in this group; there were no other incidental findings.

A low incidence of pituitary tumors was found in the female offspring only, with no differences among groups. Other incidental tumors are listed in Table 2 and were not suggestive of treatment-related effects.

Lung. Alveolar adenomas and carcinomas were common; these were pooled for statistical consideration, since not all lesions were examined histologically and distinction between benign and malignant mouse lung tumors is considered to be problematic by some pathologists. The incidence of lung tumor bearers among females in the chromium group was slightly

TABLE 3
Incidences and Multiplicities of Primary Lung Tumors

	Group 1, urethane			Group 2, $CrCl_3$			Group 3, control		
	F	M	All	F	M	All	F	M	All
No.[a]	75	52	127	71	72	143	69	47	116
No. with tumor[b]	46 (61)	28 (54)	74 (58)	50 (70)[c]	43 (60)	93 (65)[d]	37 (54)[c]	26 (55)	63 (54)[d]
Av. multiplicity ±SE	1.8 ± 0.3[e]	1.5 ± 0.3		2.0 ± 0.3[e]	1.6 ± 0.2		1.1 ± 0.2[e]	1.6 ± 0.4	
Av. size ±SE (mm)	3.0 ± 0.3	3.0 ± 0.3		2.0 ± 0.2	3.0 ± 0.3		3.0 ± 0.3	3.0 ± 0.4	

Note. All lung tumors 1.0 mm in widest dimension or larger were included.
[a] No. of lungs included; those with metastatic neoplasia were omitted because this obscures the primary pulmonary lesions.
[b] Values in parentheses are percentages.
[c] $p = 0.055$, two-tailed FET.
[d] $p = 0.052$, one-tailed FET.
[e] $p = 0.06$, Kruskal–Wallis nonparametric ANOVA; Group 2 vs Group 3, $p = 0.023$, Mann–Whitney U test; $p = 0.017$, Wilcoxon rank sum test.

greater than in controls, 70% vs 54%, a difference of borderline significance (Table 3). Average multiplicity of lung tumors was approximately double that in controls, a difference of statistical significance. This difference was apparent for all cohorts of mice dying between 700 and 1100 days of age ($p = 0.004$) (Fig. 2). There was a lesser increase in lung tumor multiplicity in the female offspring of the urethane group. Average tumor sizes were similar among groups and sexes.

Because of the advanced age of most of the mice with lung tumors, we considered the possibility that some of the smaller tumors could have been derived secondarily from local spread of primary lung tumors. To test for this, we carried out the statistical analysis omitting outliers (mice with more than three times the average number of lung tumors); the difference between Groups 2 and 3 was still significant ($p = 0.031$, Mann–Whitney test). We also analyzed the data including only tumors 2 mm in size or larger. A difference between Groups 2 and 3 was still seen, though of lesser significance because of the lower N value (data not shown).

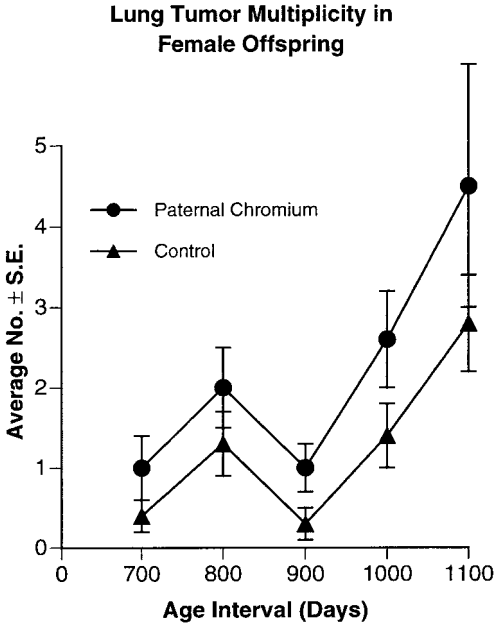

FIG. 2. Average number of lung tumors in the mice dying during the indicated intervals. ●, paternal chromium(III) treatment; ▲, controls. The numbers of individuals represented are <700 days, $N = 16$; 700–800 days, $N = 18$; 800–900 days, $N = 12$; 900-1000 days, $N = 17$; 1000–1100 days, $N = 8$. $p = 0.0042$ (ANOVA), for treatment-related effect.

Non-neoplastic findings in lungs were lymphocytic infiltrates, alveolar cell hyperplasia, and, less frequently, edema, hemorrhage, macrophage presence, and inflammation. No attempt was made to correlate these changes with presence of tumors.

Hematopoietic neoplasms. The most common hematopoietic neoplasms were lymphomas and histiocytic sarcomas, especially in females (Table 4). Lymphomas were most frequent in urethane-group female offspring; five occurred in these mice before 400 days. Lymphomas were less frequent in the chromium-group female offspring than in controls. The difference in lymphoma incidence between the females of the two treatment groups was significant and was apparent at all ages (Fig. 3) ($p = 0.055$, Cox's test, and $p = 0.0307$ generalized Kruskal–Wallis analysis of death-from-lymphoma data). By contrast, the urethane-group females did not develop any histiocytic sarcomas, a difference of statistical significance compared with controls. Non-neoplastic findings for lymph nodes, spleens, and thymuses included lymphoid hyperplasia, plasmacytosis, histiocytosis, cystic dilation, hemorrhage, polyarteritis nodosa, fibrosed artery, chronic inflammation, extramedullary hematopoiesis, pigment, congestion, thymic cortical atrophy, thymic medullary hyperplasia, mastocytosis, abscess, eosinophilia, and ossification. None of these occurred with high frequency or showed a relationship to treatment.

TABLE 4
Incidences of Hematopoietic Neoplasms in Offspring of Treated and Control Males

Neoplasm	Female offspring			Male offspring		
	Group 1, urethane ($N = 78$)	Group 2, $CrCl_3$ ($N = 71$)	Group 3, control ($N = 71$)	Group 1, urethane ($N = 54$)	Group 2, $CrCl_3$ ($N = 72$)	Group 3, control ($N = 48$)
Lymphoma, total[a]	21 (27)[b]	8 (11)[b]	15 (21)[b]	5 (9)	5 (7)	2 (4)
Lymphoblastic	9	3	7	0	0	0
FCC	11	5	6	5	3	2
Immunoblastic	0	0	1	0	1	0
Not specified	1	0	1	0	1	0
Histiocytic sarcoma	0 [c]	6	6 [c]	2	2	1
Mast cell tumor	2	1	1	1	1	0
Myelogenous leukemia	1	1	0	0	1	1
Thymoma	0	0	1	0	0	0
Not specified	2	3	1	1	0	1
Total[a]	26 (33)	19 (18 mice, 25)[d]	23 (22 mice, 31)[e]	9 (17)	9 (12)	5 (10)

Note. FCC, follicle center cell lymphoma; Not specified, neoplasm not categorized due to ambiguous features or autolysis.
[a] Values in parentheses are percentages.
[b] $p = 0.056$, χ^2 test for independence; $p = 0.022$, Group 1 vs Group 2, two-tailed FET.
[c] $p = 0.010$, two-tailed FET.
[d] One mouse presented both FCC lymphoma and histiocytic sarcoma.
[e] One mouse presented both thymoma and histiocytic sarcoma.

Soft-tissue neoplasms. A variety of soft-tissue neoplasms were found. There were no differences between groups of statistical significance (Table 5). However, female offspring in each treatment group had a somewhat lower incidence of all types of soft-tissue neoplasm combined, compared with controls.

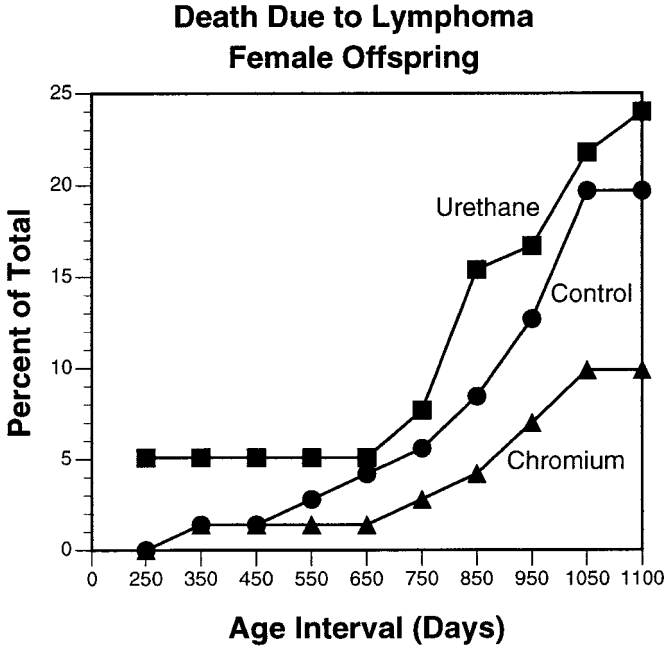

FIG. 3. Percent of total at-risk female offspring dying of lymphoma during the indicated intervals. ■, urethane group; ▲, chromium group; ●, controls. The data for Group 1, urethane treatment of fathers, and Group 2, chromium treatment of fathers, are significantly different (see text).

Female reproductive tissues. Neoplasms of mammary gland, ovary, uterus, and cervix occurred at low incidence and with no differences among groups (Table 6). Non-neoplastic changes were fairly common, and the frequency of some of these was greater in the treated-group offspring compared with controls. Increases in cystic changes in ovaries and uteri occurred after both paternal treatments, with borderline significance in some comparisons. Other incidental changes that showed no relation to treatment included, in ovaries, polyarteritis nodosa, hematocyst, corpus hemorrhagica, interstitial cell hyperplasia, and fat necrosis; in mammary glands, edema, hematocyst, polyarteritis nodosa, cystic dilation, mesovarium thrombus, granulosa cell hyperplasia, thrombosis, mineralization, and lymphocytic infiltrate; and in uteri, hematocysts.

Male reproductive tissues. Neoplasms were relatively infrequent in these tissues, with sarcomas of the seminal vesicles being those most commonly seen (Table 7), especially in the chromium-group offspring. Polyps and cystadenomas occurred in the Cowper's glands of treated-group offspring only, but were of low incidence. The incidence of any neoplasm occurring in male reproductive tissue was greater in the chromium-group males compared with controls.

Non-neoplastic changes in testes were slightly more frequent in urethane-group males, with a greater number of atrophied or small testes, and the presence of pigment in 8% of the testes. Pigment was not seen in any control male testes ($p < 0.05$) and in only one chromium-group male.

TABLE 5
Incidences of Sarcomas and Vascular Tumors

Site/type	Female offspring			Male offspring		
	Group 1, urethane ($N = 78$)	Group 2, $CrCl_3$ ($N = 71$)	Group 3, control ($N = 71$)	Group 1, urethane ($N = 54$)	Group 2, $CrCl_3$ ($N = 72$)	Group 3, control ($N = 48$)
All[a]	12 (15)	11 (15)[b]	17 (24)[c]	8 (15)[d]	16 (22)	6 (12)
Hemangioma	1	1	0	1	0	0
Hemangiosarcoma/pericytoma	6	3	9	1	4	2
Leiomyoma	0	1	3	—	—	—
Leiomyosarcoma	2	4	0	—	—	—
Endometrial stromal sarcoma	0	1	0	—	—	—
Myxosarcoma	0	0	1	0	0	0
Fibrosarcoma	1	1	0	0	1	0
Osteosarcoma	0	0	1	0	0	0
Undifferentiated sarcoma	1 cecum, 1 sc	1 cecum, 1 sc	1 head, 1 muscle, 1 mediastinum, 2 sc	1 pancreas, 5 seminal vesicles, 1 sc	1 cecum, 10 seminal vesicles	4 seminal vesicles

[a] Values in parentheses are percentages.

Co-occurence in the same mouse: [b] sarcoma of cecum and leiomyoma; hemangiosarcoma and leiomyosarcoma; [c] hemangiosarcoma and leiomyoma or myxosarcoma; [d] hemangioma and sarcoma in seminal vesicles.

Abnormalities which occurred but showed no apparent difference among groups were testicular cysts, dilation and/or chronic inflammation of the coagulating gland, dilation and/or subacute inflammation of the preputial gland, and ulceration of the seminal vesicles (data not shown). Bilateral abnormality of testis was slightly higher in treated groups.

Male liver. A few neoplasms occurred, unrelated to treatment (Table 8). Assorted non-neoplastic changes were noted, generally at higher incidence in the livers of the treated-group male offspring compared with controls. The frequency of any hepatic abnormality in the urethane-group males, 24%, was greater than that in the control males (10%). This was related to more common occurrence of inflammation and a considerable variety of other alterations (see footnote to Table 8).

Male kidney. Neoplasms were uncommon and were found only in male offspring of treated fathers (Table 8). Non-neoplastic pathological changes, including nephropathy and cysts and others at lower frequency (see footnote to Table 8) occurred in the male offspring of chromium-treated fathers more often than in control male offspring. Male offspring of the urethane-treated fathers did not differ from controls with regard to incidence of kidney lesions.

Paternal- and litter-specific effects. Since the fathers were the unit of treatment, the data were also analyzed to assess the numbers of fathers affected by the treatments, such that a significant effect occurred in at least one offspring. Data for all changes showing a difference from the controls of at least $p = 0.05$, two-tailed test, were considered (Table 9). For female lung, four or more tumors, or twice the average multiplicity, was taken as a conservative measure. The occurrence of testicular pigment in the urethane-group males was omitted from this analysis because of the likely litter effect (see below). For five of the six changes analyzed, the results were also significantly different when the fathers were the statistical units. The exception was Harderian gland tumors, but even here a higher percentage of the treated males had offspring with a Harderian gland tumor, compared with control, although the difference was not of statistical significance.

In a further test of whether any of the significant effects noted were due primarily to a contribution from one or a few fathers or litters, tests for homogeneity of sire and litter proportions were carried out. No significant paternal effect was noted. The only significant litter effect was for the presence of pigment in testes (see Table 7), where three of the six affected males were from the same litter ($p = 0.024$).

Percent of all offspring affected by paternal treatments. The treatment-related effects in the offspring which were significant by the most conservative statistical criteria ($p < 0.05$ by two-tailed tests for both individual offspring and for fathers, with no litter effects) were tumors of the adrenal gland, preneoplasia/neoplasia of the glandular stomach (males, urethane group), lung tumors (females, chromium group), and bilateral kidney change (males, chromium group). The percentage of all male offspring affected by one or more of these changes was increased significantly from 3% in controls to 15% in the urethane group and 14% in the chromium group (Table 10). Chromium-group females also showed a significant increase, 7% to 21%. Changes that appeared likely to be related to treatment, when somewhat less stringent criteria were applied (see footnote to Table 10), included thyroid tumors (chromium group), Harderian gland tumors (males of both groups), lymphomas (females, urethane group), lung tumors (females, ure-

TABLE 6
Lesions in the Reproductive System of Female Offspring

Tissue	Group 1, urethane (N = 78)	Group 2, $CrCl_3$ (N = 71)	Group 3, control (N = 71)
Mammary, any lesion	10 (13)	6 (8)	9 (13)
Neoplasms	6 (5 AC, 1 ACS) (8)	5 AC (7)	5 (4 AC, 1 ACS) (7)
Hyperplasia/metaplasia	4/1	2/0	3/0
Ovary, any lesion	18 (23)	19 (27)	14 (20)
Neoplasms	3 (1 GCT, 1 luteoma, 1 HMS) (4)	1 GCT (1)	5 (1 luteoma, 4 HMS) (7)
Cysts	12 (15)[a]	13 (18)[b]	5 (7)[a, b]
Atrophy	9 (12)	8 (11)	7 (10)
Uterus, any lesion	44 (56)[c]	35 (49)	29 (41)[c]
Neoplasms	5 (1 endometrial Ad, 1 endometrial CA, 2 LMS, 1 HMS) (6)	9 (1 endometrial stromal polyp, 1 stromal sarcoma, 1 LM, 3 LMS, 2 HMS, 1 FS) (13)	8 (1 endometrial AC, 1 stromal polyp, 3 LM, 3 HMS) (11)
Cystic endometrial hyperplasia	24 (31) 2.3 ± 0.6[e]	24 (34)[d] 2.7 ± 0.6[e]	15 (21)[d] 2.3 ± 0.9[e]
Dilation	8 (10)	6 (8)	3 (4)
Angiopathy	16 (21)	7 (10)	10 (14)
Cervix, neoplasms	1 HMS (adjacent)	1 LMS	None

Note. Values in parentheses are percentages. AC, adenocarcinoma; ACS, adenocarcinomasarcoma; GCT, granulosa cell tumor; LMS, leiomyosarcoma; HMS, hemangiosarcoma; LM, leiomyoma; FS, fibrosarcoma.

[a] $p = 0.089$, one-tailed FET.

[b] $p = 0.075$ and 0.038, two- and one-tailed FET, respectively.

[c] $p = 0.071$ and 0.041, two- and one-tailed FET, respectively.

[d] $p = 0.066$, one-tailed FET.

[e] Average severity score ± SD.

thane group), and hepatic alterations (males of both groups). When offspring showing one or more of any of these changes were totaled, there were significant increases for males of both the urethane group (36%) and the chromium group (39%) compared with control males (20%), and marginal increases for the urethane-group females (30% to 44%) and the chromium-group females (10% to 23%). Thus, between 10% and 20% percent of offspring were affected by the paternal treatments, depending on chemical agent, sex, and statistical criteria used.

Correlations among effects due to preconception exposure. All data were examined carefully for possible correlations: animals showing any of the possibly significant changes were grouped and scanned for other changes occurring frequently. A multiplicity of three or more lung tumors in the females was used as the indicator (instead of four as above) to increase statistical power. Data were analyzed by the Fisher exact test. Seven correlations were discovered, as listed in Table 11, three for the urethane treatment group and four for the chromium treatment group. In two cases, a significant change correlated with a parameter not showing a treatment-related difference: presence of testicular pigment with absence of lung tumors in the urethane-group males and pheochromocytomas with pituitary tumors in the chromium-group females.

Because of the large number of comparisons made, we considered whether these correlations could have occurred entirely by chance. In all, comparisons were made among 10 female tissues or neoplasms (adrenal, thyroid, lung, Harderian gland, pituitary, uterus, ovary, lymphoma, sarcoma, and histiocytic sarcoma) and 12 male tissues, abnormalities, or neoplasms (adrenal, thyroid, Harderian gland, lung, sarcoma, testicular atrophy, testicular pigment, seminal vesicles, Cowper's gland, liver, kidney, and stomach). Thus, the number of comparisons made was 111 (45 for females and 66 for males), based on the formula $n(n - 1)/2$. We observed six correlations at a significance level of $p = 0.03$ or lower (Table 11), whereas three would have been expected by chance. Application of the binomial expansion formula to these data produced a probability of approximately 0.12 that 6 or more of these 111 correlations would have attained significance at the $p = 0.03$ level by chance alone. In the case of the females, 5 of the 45 correlations were significant at the $p = 0.05$ level; the binomial expansion with $N = 45$ and $p = 0.05$ gives a probability of approximately 0.07 that five or more correlations would have attained significance by chance alone. Thus, it seems likely that at least some of these correlations were not due entirely to chance.

Oxidative DNA damage in testes of chromium-exposed males. As a test of whether a mechanism of action of chromium in the testes could include oxidative DNA damage, DNA from whole testis was analyzed for 8-oxo-dG at varying times

TABLE 7
Lesions in Reproductive Organs of Male Offspring

Tissue	Group 1, urethane ($N = 75$)	Group 2, $CrCl_3$ ($N = 92$)	Group 3, control ($N = 86$)
Testes, any lesion	31 (41)[a]	32 (35)	26 (30)[a]
Atrophy/small size	29 (39)	29 (32)	23 (27)
Pigment	6 (8)[b]	1 (1)	0[b]
Mineralization	8 (11)	8 (9)	4 (5)
Seminal vesicles, any lesion	37 (49)	51 (55)	41 (48)
Neoplasms	6 (5 sarcomas, 1 hemangiosarcoma) (8)	12 (10 sarcomas, 1 granular cell tumor, 1 histiocytic sarcoma) (13)[c]	5 (4 sarcomas, 1 hemangiosarcoma) (6)[c]
Dilation	27 (36)	44 (48)	33 (38)
Fibrosis	7 (9)	11 (12)	6 (7)
Chronic inflammation	21 (28)	24 (26)	16 (19)
Cowper's gland, any lesion	12 (16)	16 (17)[d]	7 (8)[d]
Neoplasms	1 polyp (1)	3 (1 polyp, 2 cystadenomas) (3)	0
Dilation	11 (15)	12 (13)	7 (8)
Prostate	0	0	1 adenoma
Any male reproductive organ neoplasm	7 (9)	15 (16)[e]	6 (7)[e]

Note. Numbers in parentheses are percentages.
[a] $p = 0.096$, one-tailed FET.
[b] $p = 0.0091$, two- and one-tailed FET.
[c] $p = 0.082$, one-tailed FET.
[d] $p = 0.076$ and 0.052, two- and one-tailed FET, respectively.
[e] $p = 0.064$ and 0.044, two- and one-tailed FET, respectively.

after administration of the same dose of $CrCl_3$ as in the carcinogenicity study. No differences were noted (Table 12).

DISCUSSION

The results of this study provide confirmation of the fact that exposure of male mice to either urethane or chromium(III) results in multiple pathological alterations in the progeny, including changes in tumor incidence. Many different tissues were affected, including adrenal gland, glandular stomach, lung, and kidney by the most conservative statistical criteria, and also probably thyroid, Harderian gland, lymphoid tissue, liver, uterus, ovary, and male reproductive glands. This demonstration of such diverse significant effects, involving 10% to 20% of the offspring, with almost all exposed fathers producing offspring showing at least one of these effects, is without precedent. Only three published reports include complete findings for preconception-exposed offspring living until natural death, two involving radiation exposure of male mice. Male C3Hf mice were treated with X-rays 7 weeks before mating, so that spermatogonia were exposed (Cosgrove *et al*., 1993), rather than spermatids as in our study. No changes in survival were noted and the authors concluded that no treatment-related pathological changes occurred, although our further analysis of their data reveals possible decreased tumor incidences in females, for lung ($p = 0.039$) and leukemia/lymphoma ($p = 0.062$). In the other radiation study, C57BL/6 mice were exposed to gamma radiation 15 days before mating, a timing protocol similar to ours (Iwasaki *et al*., 1996). The only significant effect reported was a decrease in histiocytic sarcomas in female offspring, as in the female progeny of urethane-treated males in our study. Our further analysis of the data in this article shows a possible increase in hepatocellular carcinoma in the male offspring ($p = 0.059$). In a similar experiment by the same investigators, neutron irradiation of C3H male mice, 2 weeks before mating with C57BL/6 females, led to a large, highly significant increase in liver tumors in male offspring at 14.5 months (43% vs 3% in controls); irradiation 3 months before mating had a much smaller, nonsignificant effect (Watanabe *et al*., 1996).

In the third study, male CBA mice were exposed transplacentally to diethylstilbestrol and then mated as adults (Walker, 1984). Among their female offspring, significant increases occurred in uterine sarcomas, lymphoma, and ovarian tumors, compared with controls. These several studies, together with ours, illustrate that preconception carcinogenic effects vary widely with regard to degree of effect and nature of the target tissues, as a function of the exposure agent, mouse strain, and/or time of treatment.

An interesting outcome in our study was the appearance of pheochromocytomas in the offspring of both treatment groups, with none in the controls. The adrenal gland has not previously been reported as a target for preconception carcinogenesis. The incidence of these tumors of the adrenal medulla was low, 4% to 7%; however, non-neoplastic abnormalities also increased in

TABLE 8
Incidence of Lesions in Male Liver and Kidney

Tissue	Group 1, urethane ($N = 74$)	Group 2, $CrCl_3$ ($N = 92$)	Group 3, control ($N = 86$)
Liver, any lesion	18 (24)[a]	18 (20)[a]	9 (10)[a]
Neoplasms/preneoplastia	0	4 (1 histiocytic sarcoma, 3 preneoplastic foci) (4)	1 hepatocellular adenoma (1)
Inflammation	9 (12)[b]	5 (5)	3 (3)[b]
Other[c]	14 (19)[c]	15 (16)[c]	4 (5)[c]
Kidney, any lesion	9 (12)[d]	22 (24)[d]	10 (12)[d]
Neoplasms	2 (3)[e]	2 (2)[f]	0
Bilateral lesions	2 (3)[g]	12 (13)[g]	3 (3)[g]
Nephropathy	6 (8)	14 (15)[h]	6 (7)[h]
Bilateral	2 (3)[i]	6 (7)[i]	0 [i]
Unilateral	4	8	6
Cyst	7 (9)[j]	16 (17)[j]	6 (7)[j]
Bilateral	1	4	2
Unilateral	6 (8)	12 (13)[k]	4 (5)[k]
Other[l]	11 (15)[l]	12 (13)[l]	4 (5)[l]

Note. Values in parentheses are percentages.

[a] $p = 0.064$, χ^2 test for independence; Group 1 vs Group 3, $p = 0.033$, two-tailed FET.

[b] $p = 0.067$ and 0.037, two- and one-tailed FET, respectively.

[c] Included pigmented macrophages, lymphocytic infiltrates, extramedullary hematopoiesis, hepatocellular hypertrophy, coagulative necrosis, hepatocellular necrosis and bilateral cysts. Group 1 vs Group 3, $p = 0.0054$; Group 2 vs Group 3, $p = 0.014$, two-tailed FETs.

[d] $p = 0.045$, χ^2 test for independence; Group 2 vs Group 3, $p = 0.050$, two-tailed FET.

[e] 1 adenoma, 1 carcinoma.

[f] 1 adenoma, 1 sarcoma.

[g] $p = 0.010$, χ^2 test for independence; Group 2 vs Group 3, $p = 0.029$, two-tailed FET.

[h] $p = 0.099$ and 0.065, two- and one-tailed FET, respectively.

[i] $p = 0.044$, χ^2 test for independence; Group 2 vs Group 3, $p = 0.029$ and 0.018, two- and one-tailed FET, respectively.

[j] $p = 0.076$, χ^2 test for independence; Group 2 vs Group 3, $p = 0.041$ and $= 0.029$, two- and one-tailed FET, respectively.

[k] Group 2 vs Group 3, $p = 0.066$ and 0.043, two- and one-tailed FET, respectively.

[l] Included hydronephrosis, periarteritis, thickened glomerular tufts, hypertrophy, lymphocytic infiltrates, dilated tubules, and hyaline droplets. $p = 0.076$, χ^2 test for independence; Group 1 vs Group 3, $p = 0.032$ and 0.026, two- and one-tailed FET; Group 2 vs Group 3, $p = 0.066$ and 0.043, two- and one-tailed FET, respectively.

females, affecting up to 18% of the female offspring. While pheochromocytomas are fairly common in rats (Cheng, 1980), they are a rare spontaneous or induced tumor in mice. Most studies, including two with Sencar and CD-1 Swiss mice, found an incidence of 0–2% (Tischer and Sheldon, 1996); no pheochromocytomas were reported in more than 400 NIH Swiss mice maintained until natural death (Kelloff *et al*., 1976). However, higher incidences have been seen in certain genetic situations, 5–6% in females of C3HMTV− and B6C3F1 strains and 31% of females from a cross of strain I males with C3H females (Cheng, 1980). Genetic manipulations also can cause an increase in these tumors: pheochromocytomas occurred in 22% of mice heterozygous for a germline mutation in the *neurofibromatous type 1* gene (Jacks *et al*., 1994) and were also found at high incidence in some though not all lines of c-*Mos* transgenic mice (Schulz *et al*., 1992).

Although pheochromocytomas have not been reported as a consequence of chemical carcinogen exposure of mice, it is of particular interest, in view of the controversy surrounding the effects of human paternal irradiation, that the appearance of pheochromocytomas was among the delayed effects in mice exposed to atomic bomb radiation (Upton *et al*., 1960). Although it has not been determined whether mouse pheochromocytomas produce epinephrine, their cytological and histochemical characteristics suggest that this is the case; these lesions appear to be better models for human pheochromocytomas than are the corresponding rat tumors (Tischler and Sheldon, 1996; Tischler *et al*., 1996). Aging-related non-neoplastic changes have been noted in the adrenal glands of mice, including lipogenic pigment cells and hyperplasia as in our study (Tischler and Sheldon, 1996; Yarrington, 1996).

These results suggest an impact of preconception exposure on the functioning of the neuroendocrine axis. Overall incidence of pituitary tumors was not altered by treatment, but a significant correlation of occurrence of pituitary tumors with pheochromocytomas was shown in the females of the chromium treatment group. There was a possible increase in thyroid tumors, to an incidence similar to that of pheochromocytomas, 6–7% in all treatment groups except for the males of the urethane group. In other mouse studies, the spontaneous fre-

TABLE 9
Incidence of Fathers with Offspring Showing Significant Effects of Preconception Exposure

Lesions	Group 1, urethane	Group 2, $CrCl_3$	Group 3, control	Statistical tests, two-tailed	
				χ^2 (2 *df*)	FET
Pheochromocytomas, males and females	4 (40)	7 (64)	0	$p = 0.0065$	$p = 0.0039$, Gr. 2 vs Gr. 3 $p = 0.035$, Gr. 1 vs Gr. 3
Harderian gland tumors, males	6 (60)	8 (73)	5 (45)	NS[a]	NS
Glandular stomach tumors or severe hyperplasia, males	6 (60)	1 (9)	2 (18)	$p = 0.023$	$p = 0.08$, Gr. 1 vs Gr. 3
Four or more lung tumors, females	6 (60)	8 (73)	2 (18)	$p = 0.028$	$p = 0.03$, Gr. 2 vs Gr. 3 $p = 0.08$, Gr. 1 vs Gr. 3
Bilateral kidney lesions, males	2 (20)	8 (73)	3 (27)	$p = 0.026$	$p = 0.086$, Gr. 2 vs Gr. 3 $p = 0.03$, Gr. 1 vs Gr. 2
Any liver lesions, males	9 (90)	9 (82)	5 (45)	$p = 0.051$	$p = 0.063$, Gr. 1 vs Gr.3

Note. Values are the number of fathers with at least one offspring with the lesions listed. Effective number of fathers was 10 for Group 1 and 11 for Groups 2 and 3. Values in parentheses are percentages.

[a] NS, not significant.

quency of thyroid adenomas has been about 1% (Heath and Frith, 1983), and carcinomas, of which we found two, are even more rare. It is of interest that an exception was C3HMTV− females, with an incidence of 7.3%; this same strain and sex had an unusually high incidence of pheochromocytomas (see above). Experimental causation of thyroid tumors in mice by chemicals generally involves interference with thyroid hormone homeostasis and increased production of thyroid-stimulating hormone (Thomas and Williams, 1996; Williams, 1995).

An apparent increase in Harderian gland tumors was seen in our study, especially in male offspring. The incidence in control males, 6%, was similar to that reported for male Swiss NMRI mice, 4.6% (Krinke *et al*., 1996). Murine Harderian glands are light-responsive and produce a lipoidal secretion by exocytosis (Buzzell, 1996). Tumors may be caused by nongenotoxic as well as mutagenic carcinogens (Krinke *et al*., 1996).

Reproductive tissues might also be expected to be affected by changes involving the neuroendocrine axis. In the female offspring, neoplasms of the mammary gland, ovary or uterus/cervix were not altered in incidence by paternal exposure. There were possible increases in ovarian cysts and in cystic endometrial hyperplasia in both treatment groups; both are common findings in aging mice. These changes may be related to endocrine alterations, as they can be caused by administration of steroid hormones such as DES (Maekawa and Yoshida, 1996).

Possible effects on reproductive tissues were also seen in the chromium-group males, including an increase in neoplasms of the seminal vesicles (mainly sarcomas) and any change (neoplasms plus dilation) in Cowper's glands. Incidence of neoplasms in any male reproductive tissue in this group approached statistical significance when compared with controls ($p = 0.06$, two-tailed test). A highly significant ($p < 0.01$) difference was the occurrence of pigment in the testes of 8% of the urethane-group males; this was rare (1%) in the chromium group males and absent from the controls. Testicular pigmentation, due to accumulation of lipofuscin, is strain-specific in mice; none was observed in more than 2000 CD-1 control males (Gordon *et al*., 1996). In our study, a litter effect was noted for testis pigment, which may suggest a contribution of maternal genetics.

Lung tumors have been the most commonly studied tumor end point in transgenerational studies; most investigations have terminated at 1 year of age or less. A discrepancy was recently reported between effects of parental exposure of ICR Swiss mice to X-ray, with an increase in lung tumors in their offspring (Nomura, 1989, 1982) and lack of effect of the same X-ray treatment given to paternal BALB/c or C3H mice (Cattanach *et al*., 1995, 1998). Our results suggest that preconception exposure effects on lung tumor incidence may be highly mouse-strain specific. Urethane, repeatedly shown to be a strong preconception carcinogen for the ICR Swiss mice studied in Japan (Nomura, 1989, 1978, 1982), had less of an effect than chromium in our study, with only a minimal increase in multiplicity of lung tumors in female offspring. This was not due to ineffectiveness of the urethane treatment, as effects were seen on other tissues, some of them pronounced (see above).

TABLE 10
Percent Offspring Affected by Paternal Treatment

Treatment-related effects in any of the tissues below	Group 1, urethane		Group 2, chromium		Group 3, control	
	Females ($N = 78$)	Males ($N = 74$)	Females ($N = 71$)	Males ($N = 92$)	Females ($N = 71$)	Males ($N = 86$)
Significant by conservative criteria[a]: adrenal gland; glandular stomach (males, Gr. 1); lung (females, Gr. 2); kidney, bilateral (males, Gr. 2)	3 (4)[b]	11 (15)[c]	15 (21)[b]	13 (14)[c]	5 (7)[b]	3 (3)[c]
Includes above, plus other probably significant effects[d]: thyroid (Gr. 2); Harderian gland (males); lymphoid (females, Gr. 1); lung (females, Gr. 1); liver (males)	34 (44)[e]	27 (36)[f]	16 (23)[e, g]	36 (39)[f]	21 (30)[e] 7 (10)[g]	17 (20)[f]

Note. Values in parentheses are percentages.
[a] Includes changes of statistical significance $p < 0.05$ by two-tailed test for both offspring and fathers.
[b] $p = 0.0014$, χ^2 test for independence; Group 2, vs Group 3, $p = 0.028$, two-tailed FET.
[c] $p = 0.028$, χ^2 test for independence; Group 1 vs Group 3, $p = 0.022$, and Group 2 vs Group 3; $p = 0.017$, two-tailed FETs.
[d] Includes changes of statistical significance of $p < 0.05$ by one-tailed test plus other indicators (see relevant tables).
[e] $p = 0.019$, χ^2 test for independence; Group 1 vs Group 3, $p = 0.090$ and $= 0.054$, two- and one-tailed FET, respectively.
[f] $p = 0.012$, χ^2 test for independence; Group 1 vs Group 3, $p = 0.022$, and Group 2 vs Group 3, $p = 0.0054$, two-tailed FETs.
[g] $p = 0.067$ and 0.033, two- and one-tailed FET, respectively, Group 2 vs Group 3, where only thyroid effects are included as additional probably significant changes in Group 3 females.

Our previous demonstration (Anderson *et al*., 1994) of a preconception exposure effect of chromium(III) chloride on lung tumors in mouse progeny was confirmed by the present study. A possible small increase in these tumors was seen in the male offspring at 12 months of age, though not a difference of statistical significance. For the mice at natural death, a significant effect was demonstrated only in females. This was evident from both incidence and multiplicity data, was seen in all age groups, and was of statistical significance by several tests. However, the increases in lung tumor numbers were not large in magnitude. Thus, this tumor is not a convenient endpoint for quantifying preconception carcinogenic effects in NIH Swiss mice.

Hematopoietic neoplasms were common in our study, especially in the female offspring. A notable finding was the absence of histocytic sarcomas in the urethane group female offspring ($p = 0.01$ compared with the chromium group or the controls). A significant decrease in histiocytic sarcomas in female mouse offspring was also seen after paternal exposure to neutron radiation (Iwasaki *et al*., 1996). An increase in lymphomas occurred in the urethane group females in our study, whereas lymphomas were less frequent in the chromium group females than in controls; the difference between the urethane and chromium group females was of statistical significance. The incidence of spontaneous hematopoietic neoplasms varies among mouse strains and substrains; see Frith *et al*. (1996) for a summary. Several studies have shown variation in these values as a function of food restriction and body weight. Since levels of circulating hormones have been shown to be altered in such studies (Kritchevsky, 1995), endocrine changes are again implicated.

In male kidney, non-neoplastic changes were clearly associated with paternal exposure to chromium. These included bilateral changes, nephropathy, and cysts. Cystic kidneys and hydronephrosis may be congenital, especially if bilateral, and have been reported after a variety of chemical treatments, including steroid hormones and xenobiotics with hormone-like properties (Wolf and Hard, 1996). Thus, these changes in the kidneys are consistent with involvement of the endocrine system.

Adenomas, carcinomas, and atypical hyperplasias of the glandular stomach had a significantly elevated frequency in the male offspring of the urethane-treated fathers. Such lesions are quite rare in mice; an incidence of 0.1% was reported in a 2-year study of male CD-1 Swiss mice (Maekawa *et al*., 1996). No gastric adenocarcinomas were found in more than 400 control NIH Swiss mice (Kelloff *et al*., 1976). They are however common in humans and in mice can be induced by chemical carcinogens (Odashima, 1979).

Thus, current results confirm our previous finding (Anderson *et al*., 1994) that chromium(III) is a preconception carcinogen in mice. In the present study, the exposure of fathers to this metal had positive effects on tumor frequency in multiple tissues of the offspring in old age, including adrenal gland in both sexes and lung in females, and probably also thyroid, Harderian gland, and male reproductive glands. Increases in non-neoplastic changes occurred in liver and kidney in males, and probably in uterus and ovary in females. While urethane

TABLE 11
Correlations of Abnormalities Associated with Preconception Exposure

Group and Sex	Correlation	*p* value
Group 1, male	Testicular pigment and absence of lung tumors	0.0089
Group 1, female	Pheochromocytoma with thyroid tumor	0.033
Group 1, female	Lymphoma with sarcoma	0.028
Group 2, male	Seminal vesicle neoplasm with any kidney change	0.014
Group 2, female	Pheochromocytoma with pituitary tumor	0.026
Group 2, female	Three or more lung tumors with ovarian cysts	0.019
Group 2, female	Harderian gland tumors with lymphoma	0.053

Note. p values are from the two-tailed FET.

treatment also led to some of these alterations, there were clear distinctions between the consequences of paternal treatment with these two chemicals for some tissues, with exposure of fathers to urethane increasing and that to chromium decreasing the incidence of lymphomas in female offspring. Only urethane caused an increase in preneoplasia/neoplasia of glandular stomach and testicular pigment in males and a decrease in histiocytic sarcomas in females. Thus, the preconception carcinogenic effects of chromium(III) and urethane cannot be ascribed to nonspecific toxic actions in the fathers.

While urethane is a known carcinogen and DNA-damaging agent, the effects of chromium(III) are more surprising, as this ion is quite nontoxic and has been thought to be minimally or noncarcinogenic (Cohen *et al*., 1993). However, the biological ineffectiveness of chromium(III) has been related to inability to cross cell membranes; chromium(III) formed intracellularly as a result of reduction of other chromium species is highly reactive with DNA (Cohen *et al*., 1993). It is possible that target cells in the testes are unusually vulnerable to chromium(III) penetration; radiolabeled chromium(III) was observed to accumulate strongly in the interstitial tissues of mouse testis (Danielsson *et al*., 1984), and chromium(III) as well as chromium(VI) drinking-water treatment of male mice impacted negatively on fertility (Elbetieha and Al-Hamood, 1997). Trivalent chromium was more toxic to rabbit testes than hexavalent chromium, causing degenerative changes in the seminiferous tubules (Behari *et al*., 1978). Chromium(III) can mediate oxidative DNA damage (Tsou *et al*., 1996), but we found no increase in 8-oxo-dG in DNA extracted from whole testis. Further studies with germ cells are required. Recently, certain chromium(III)-DNA adducts have been found to be mutagenic (Voitkun *et al*., 1998); these may have contributed to our observed effects. In any event, our findings are consistent with increased risk of cancer at multiple targets, including kidney for Wilms' tumor, in offspring of fathers exposed occupationally to metals.

Our findings are consistent with the starting hypothesis, that preconception carcinogenesis is mediated via stimulation of spontaneous neoplasms, and with changes in gene expression as a mechanism. Most of the alterations we found in offspring as a result of preconception exposures were ones that occurred spontaneously in controls, but at a lower incidence, with pheochromocytomas and testicular pigment being the main exceptions. Second, non-neoplastic abnormalities common in aging mice were also affected. Third, some significant decreases in pathological change occurred, which could be expected if change(s) in gene expression are a mechanistic component.

The tissues affected or possibly affected by preconception exposure were either secretory (adrenal medulla, thyroid, Harderian gland, glandular stomach, type 2 cells of lung, lymphocytes, seminal vesicles, Cowper's gland, testis, and ovary) or showed abnormal secretory activity (uterus and kidney). This common feature of the diverse targets may provide a clue as to genes involved.

With regard to molecular mechanism, few studies have examined tumors or tissues in offspring after preconception exposure for relevant molecular or biochemical changes. Skin tumors promoted by 12-*O*-tetradecanoylphorbol-13-acetate in the second generation mice after transplacental exposure to 7,12-dimethylbenz[*a*]anthracene did not present H-*ras* mutations, though the latter were found in the skin tumors of their parents (Loktionov *et al*., 1992). The incidence of affected offspring in our study was similar to that reported earlier by Nomura, and, as noted by him (Nomura, 1987), is much higher than predicted from mutation results at known loci in mice. In his studies of tumors arising after paternal irradiation, cytogenetic changes were not found, and amplification of several oncogenes occurred only sporadically in transplanted tumors (Nomura, 1989). The mechanism of preconception carcinogenesis may be novel, and if so may well have unusual toxicant– and dose–response characteristics, for example, in human stud-

TABLE 12
Levels of 8-oxo-dG in Testes of Mice after Exposure to Chromium Chloride

	8-oxo-dG/10^5 dG	
Days after treatment	Chromium-treated	Control
1	1.10 ± 0.08	1.18 ± 0.13
7	1.30 ± 0.11	1.33 ± 0.22
14	1.32 ± 0.19	1.41 ± 0.17
21	0.96 ± 0.09	1.21 ± 0.09
28	1.32 ± 0.08	1.37 ± 0.25
35	1.74 ± 0.18	1.53 ± 0.14

Note. Values are means ± SE. Each sample consisted of 4–6 testes removed from Swiss male mice at the indicated times after ip injection with 1 mmol/kg $CrCl_3$.

ies, a dose-dependent effect of paternal cigarette smoking on cancer risk in offspring (Sorahan *et al*., 1997; Ji *et al*., 1997), but lack of effect thus far on offspring of fathers exposed to high doses of radiation from the atomic bomb (Yoshimoto, 1990) or the Chernobyl accident (Petridou *et al*., 1996).

Elucidation of the underlying molecular mechanism may assist in understanding and management of these risks. At present, one can only speculate as to the genes involved, and the means by which they are altered. Involvement of the ras signaling pathway is suggested by the increased incidences of pheochromocytomas, thyroid follicular cell tumors, and Harderian gland tumors. Pheochromocytomas are common in mice with targeted disruption of *Nf1*, which codes for a ras p21 GAP (Jacks *et al*., 1994). Follicular thyroid cancers in humans, corresponding to those found in our mice, frequently present mutated K-*ras* (Williams, 1995). Harderian gland tumors in mice have a high frequency of both H- and K-*ras* mutations (Hong *et al*., 1997) and may be caused by transgenic overexpression of N-*ras* (Coto-Montes *et al*., 1997). Overexpression of transforming growth factor alpha, which activates the ras pathway, led to hyperplasia and tumors in the glandular stomach in transgenic mice (Tamano *et al*., 1995). K-*ras* mutations are frequently found in mouse lung tumors (Malkinson, 1992). Thus, all of the prominent neoplastic effects of preconception exposure in our study could, potentially, be a consequence of abnormal functioning of the ras signaling pathway. Whether this is in fact the case and whether genetic or epigenetic alterations underlie the effects require further experimentation.

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