

## Effect of hormone treatment on spontaneous and radiation-induced chromosomal breakage in normal and dwarf mice

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### Summary

Treatment of dwarf mice with growth hormone, insulin and testosterone had no effect on the spontaneous frequencies of micronuclei (MN) in bone-marrow cells, whereas thyroxine decreased these frequencies. The induction of MN by X-rays and mitomycin C was significantly lower in dwarf mice than in normal mice. Treatment with thyroxine plus growth hormone restored normal radiosensitivity in dwarfs.

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Gonadotropic hormones and oral contraceptives are frequently used for therapeutic or experimental induction, respectively, of inhibition of ovulation in human and other mammals. During the last few years, various aspects of mutagenesis of these substances have been studied (Elbling, 1975; Bishun et al., 1976, for review).

The recent introduction of many other hormones taken continuously for a relatively long period of time for therapeutic use by patients suffering from endocrine disorders, has raised the problem of possible mutagenic effects of such therapies. However, only limited data are available on mutagenic effects of hormones influencing cell multiplication, such as growth hormone (GH) and thyroxine (Duca-Marinescu and Negoescu, 1973; Duca-Marinescu and Simionescu, 1973; Nilsson et al., 1975). The dwarf mouse, discovered in 1929 by Snell, shows many similarities with panhypopituitarism in man. As in man, the severe growth retardation, which becomes apparent 2 weeks after birth, is partially restored by hormonal treatment (van Buul-Offers and van den Brande, 1978, 1979). The similarity of this animal model with the clinical situation in humans prompted us to study the frequency of

chromosomal aberrations in Snell dwarf mice after treatment with GH, thyroxine, testosterone and insulin, using the bone-marrow micronucleus test.

It is now well established that types and frequencies of radiation-induced chromosomal aberrations are strongly cell cycle-dependent (Evans, 1962; Kihlman, 1977). Since there are indications that GH and thyroxine have an effect on the cell cycle of bone-marrow cells (Bardik, 1969; Romanov et al., 1969; Romanov and Kasavina, 1970; and Judin and Antipenko, 1972), it seemed of interest to study the induction of chromosomal aberrations by X-rays in the above-mentioned dwarf mice before and after hormone treatment.

## Materials and methods

### *Animals*

Dwarf mice (dw/dw) and phenotypically normal Snell mice (dw/+; +/+) were bred by mating heterozygotes at the Central Animal Center, Medical School, Rotterdam. They were kept under standardized laboratory conditions (temperature 24°C, 40% humidity, 12-h day-night rhythm), as described elsewhere (van Buul-Offers and van den Brande, 1978).

Swiss random-bred male mice (Cpb(SE)S) were obtained from the Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands, and kept under normal laboratory conditions.

### *Hormones*

Human growth hormone (hGH) (2 U/mg) was a gift from AB Kabi (Stockholm). Porcine growth hormone (pGH) (1 U/mg) was obtained from Koch-Light, porcine insulin (Actrapid) from Novo, L-thyroxine ( $T_4$ ) and testosterone from Sigma.

Solutions of the hormone, except testosterone, were prepared in sterile 0.9% NaCl, pH 7.4, stored at  $-20^\circ\text{C}$  and used for 3–4 weeks. pGH and  $T_4$  were first dissolved under alkaline conditions (0.1 NaOH) in saline; the pH was adjusted to 8.0 with HCl.

Testosterone was dissolved in a few drops of 96% ethanol and suspended in sterile arachis oil (Lansberg, Rotterdam).

### *Experimental design*

To study the possible mutagenic effects of hormone treatment in vivo, male and female dwarf mice aged 6–8 weeks were treated with pGH, hGH,  $T_4$ , insulin and testosterone. The animals were injected subcutaneously in the neck during 5 days a week at around 10.00 h with 0.1 ml hormone solution at different dosages, for various lengths of time (Table 1). Injections with insulin were given 3 times a day. Control animals received 0.1 ml saline, and in the testosterone experiment 0.1 ml oil. The animals were killed by decapitation under ether anaesthesia, 2 h after the last injection. The femurs were removed and bone-marrow smears made by following the standard technique of Lederbur and Schmid (1973). For each femur, 1000 polychromatic erythrocytes were analyzed for the presence of micronuclei (MN) indicative

for chromosomal breakage. The radiation studies were performed with a Siemens Stabilipan X-ray machine operating at 240 kV and 15 mA resulting in an HVL of 3.2 mm Cu and an absorbed exposure rate of 60 rad/min at a distance of 40 cm from the source. Different doses and sampling times were used to study the induction of MN in polychromatic erythrocytes of dwarf mice. In one experiment, mitomycin C was used as a mutagenic agent. In all experiments 2 femurs per animal and 1000 polychromatic erythrocytes per femur were analyzed. Significance of differences was calculated with the Student *t* test.

## Results

### *Hormone-treated dwarf mice*

Treatment of dwarf mice with hGH, pGH and thyroxine at the dosages used in Table 1 stimulated growth of these animals, although normal values were not reached. Insulin at the doses tested had only a minimal effect on body growth, whereas testosterone had no effect at all (van Buul-Offers and van den Brande, 1978, 1979).

The normally hypothyroid dwarf mice become euthyroid by treatment with 0.1  $\mu$ g thyroxine, whereas 1  $\mu$ g thyroxine induced severe hyperthyroidism (van Buul-Offers and van den Brande, 1979).

The results of bone-marrow-smear analysis after hormone treatment of dwarf mice, are summarized in Table 1. When no significant differences, for different hormone concentrations, were found, the data were pooled. In normal as well as dwarf mice the spontaneous frequency of MN in males was significantly higher than in females, a difference that seemed to disappear at older age in the normal Snell strain. In Swiss mice this sex difference did not occur. None of the hormone treatments used induced an increase in the frequencies of MN present in polychromatic erythrocytes. In dwarf mice treated with pGH alone and hGH, the observed sex difference was maintained, in contrast, a slight but significant decrease was seen in the males after treatment with insulin, testosterone and 1  $\mu$ g thyroxine alone or in combination with hGH. Owing to this effect, sex differences are no longer apparent in these groups, except for the testosterone-treated animals.

### *Mutagen treatments*

Radiation experiments with the two mouse strains and 300 rad X-rays are presented in Table 2 as induced MN per 1000 cells. Since, with the experimental group size used, no significant differences were observed in response to irradiation, or to mitomycin C, between females and males, data for both sexes were pooled. No differences in the frequencies of induced MN in polychromatic erythrocytes of Swiss random-bred and normal Snell mice were present, with a time interval of 18 h between irradiation and sampling. In dwarf mice, lower values were obtained. The low induction of MN in dwarf mice was consistent at sampling times of 18 and 24 h. In both strains of mouse, the frequencies obtained were lower at 24 h sampling time than at 18 h. Because the developmental age of dwarf mice is grossly comparable

TABLE 1

FREQUENCIES OF MN IN BONE-MARROW CELLS OF CONTROLS AND HORMONE-TREATED DWARF MICE

Age (weeks)	Controls	Number of MN per 1000 polychromatic erythrocytes $\pm$ S.E.M.		Levels of significance, $P < 0.05$ between the sexes
		Female	Male	
9-13	+ / + (Snell)	1.2 $\pm$ 0.4 (10) <sup>a</sup>	6.1 $\pm$ 0.6 (15)	s
23			2.8 $\pm$ 0.8 (4)	
10-13	dw/dw (Snell)	3.9 $\pm$ 1.3 (12)	7.9 $\pm$ 1.3 (17)	s
18-23		1.9 $\pm$ 0.4 (12)	7.6 $\pm$ 0.9 (15)	s
10-20	+ / + (Swiss)	4.2 $\pm$ 1.1 (29)	4.9 $\pm$ 0.7 (20)	ns
Hormone treatment	dw/dw (Snell) weeks of treatment			
pGH 10 or 100 $\mu$ g <sup>b</sup> /day	16	2.5 $\pm$ 0.8 (8)		
	16		9.0 $\pm$ 1.8 (5)	s
hGH 8.3 $\mu$ g/day	4-5	1.3 (2)	5.1 $\pm$ 0.6 (8)	nt
T <sub>4</sub> 1 $\mu$ g/day	1	1.2 $\pm$ 0.5 (5)	2.5 $\pm$ 0.8 (4) *	ns
1 $\mu$ g/day	4		2.0 (2)	
0.1 $\mu$ g/day	13-16	1.2 $\pm$ 0.5 (5)	4.3 $\pm$ 2.0 (6)	ns
1 $\mu$ g/day	13	2.3 $\pm$ 1.2 (3)		
pGH 100 $\mu$ g + 0.1 $\mu$ g T <sub>4</sub> /day	16	3.0 $\pm$ 2.4 (3)	2.0 (1)	nt
hGH 8.3 or 25 $\mu$ g + 1 $\mu$ g T <sub>4</sub> /day <sup>b</sup>	10	1.3 $\pm$ 0.4 (4)	2.3 $\pm$ 1.2 (3) *	ns
Insulin 7.5 or 15 mU/day <sup>b</sup>	5	1.6 $\pm$ 0.5 (8)	3.8 $\pm$ 0.8 (5) *	ns
Testosterone				
♂ 20, 200 or 2000 $\mu$ g/day <sup>b</sup>	5	2.6 $\pm$ 0.5 (5)	4.7 $\pm$ 0.2 (10) *	s
♀ 20 or 200 $\mu$ g/day <sup>b</sup>				

<sup>a</sup> The number of animals is indicated in parentheses; from each animal 2000 cells were analyzed.<sup>b</sup> Results of different concentrations were pooled.

\* Significantly different from dwarf controls.

with 2-3-week-old normals (van Buul-Offers and van den Brande, 1981), the induction of MN in 3-week-old Swiss mice was studied. Table 2 shows the same radiosensitivity for these young mice as for adults, suggesting that the observed differences between dwarf mice and normals cannot be easily attributed to differences in radiosensitivities of various developmental ages at comparable chronological ages.

Treatment of dwarf mice with mitomycin C indicated no induction at all of MN,

TABLE 2

INDUCTION OF MN IN POLYCHROMATIC ERYTHROCYTES AFTER 300 rad X-RAYS OR 2.5 mg MITOMYCIN-C PER kg (DATA POOLED FOR BOTH SEXES; SEX RATIO 1:1)

Mouse strain	Pretreatment	Treatment	Time interval between treatment and observation (h)	Number of mice <sup>a</sup>	Induced MN/1000 $\pm$ S.E.M.
+ / + (Swiss)	—	MMC	24	6	19.7 $\pm$ 3.2
dw/dw (Snell dwarf)	—	MMC	24	6	0 $\pm$ 1.3
+ / + (Swiss)	—	X-rays	18	4	46.0 $\pm$ 5.3
+ / + (Snell)	—	X-rays	18	8	39.9 $\pm$ 4.2
+ / + (Swiss young) <sup>b</sup>	—	X-rays	18	4	46.5 $\pm$ 5.1
dw/dw (Snell dwarf)	—	X-rays	18	10	18.7 $\pm$ 3.3
+ / + (Snell)	—	X-rays	24	4	8.4 $\pm$ 1.4
dw/dw (Snell dwarf)	—	X-rays	24	2	0
dw/dw (Snell dwarf)	—	X-rays	7	2	0.2
+ / + (Snell)	NaCl 3 weeks <sup>c</sup>	X-rays	18	6	35.2 $\pm$ 5.8
+ / + (Snell)	T <sub>4</sub> + pGH 3 weeks <sup>c</sup>	X-rays	18	6	40.1 $\pm$ 6.3
dw/dw (Snell dwarf)	T <sub>4</sub> + pGH 3 weeks <sup>d</sup>	X-rays	18	10	34.3 $\pm$ 2.4
dw/dw (Snell dwarf)	T <sub>4</sub> + pGH chronically <sup>e</sup>	X-rays	18	4	30.1 $\pm$ 5.7

<sup>a</sup> From each animal, 2000 cells were analyzed.

<sup>b</sup> Mice were 3 weeks old.

<sup>c</sup> The mice received subcutaneously NaCl solution or a cocktail of 0.1  $\mu$ g thyroxine and 0.1 U pGH for 3 weeks 5 days per week, and were subsequently irradiated.

<sup>d</sup> The mice received subcutaneously a cocktail of 1  $\mu$ g thyroxine and 0.4 U pGH for 3 weeks 5 days per week, and were subsequently irradiated.

<sup>e</sup> The mice received subcutaneously a cocktail of 1  $\mu$ g thyroxine and 0.1 U pGH for 2 months (5 days per week); after 2 additional months without treatment the mice were irradiated.

whereas in Swiss mice significantly enhanced frequencies were observed. The ratio between polychromatic erythrocytes and more mature ones in bone-marrow smears, which normally should be around 1 (Schmid, 1976), was similarly affected in dwarfs and normals ( $0.48 \pm 0.07$  vs.  $0.40 \pm 0.04$ ) and indicated severe cell killing in both genotypes.

After treatment of dwarf mice for 3 weeks with a combination of pGH and thyroxine, at a concentration that stimulated body growth and caused a hyperthyroid status of the animal, the induction of MN by 300 rad X-rays reached normal values.

Irradiation of dwarf mice treated with pGH and thyroxine, followed by a period of 2 months without treatment, showed an intermediate restoration of normal radiosensitivity (Table 2). Treatment of normal Snell mice with pGH and thyroxine did not change the radiosensitivity for MN induction in polychromatic erythrocytes (Table 2).

TABLE 3  
THE INDUCTION OF MN IN POLYCHROMATIC ERYTHROCYTES AFTER VARIOUS DOSES OF X-RAYS (DATA POOLED FOR BOTH SEXES; SEX RATIO 1:1)

Dose (rad)	Time interval between irradiation and observation (h)	MN/1000 cells $\pm$ S.E.M.	
		+ / + (Swiss)	dw / dw (Snell dwarf)
—	—	2.0 $\pm$ 0.8	3.8 $\pm$ 1.3
50	18	30.0 $\pm$ 2.3	13.8 $\pm$ 2.3
50	27	26.8 $\pm$ 1.3	15.0 $\pm$ 0.7
100	18	61.8 $\pm$ 8.8	20.0 $\pm$ 2.6
100	27	54.0 $\pm$ 5.8	25.0 $\pm$ 3.0

For each point, 4 mice were used, except for unirradiated dwarf mice which consisted of 2 males and 1 female; from each animal 2000 cells were analyzed.

Results of experiments at the lower doses of 50 and 100 rad with two different sampling times are presented in Table 3. At these lower dose levels, at both sampling times, the induction of MN in bone-marrow cells of dwarf mice was clearly lower. Although the differences were non-significant, it is interesting to note that, in dwarf mice, the peak yield of MN was obtained 27 h after irradiation, but for normal mice at 18 h, suggesting some differences in formation-differentiation kinetics of pro-erythrocytes. The dose-response relationships for MN induction in dwarfs and normal mice at 18 h sampling time are plotted in Fig. 1. For normal mice a maximum was found at around 100 rad, whereas in dwarf mice such a distinct peak

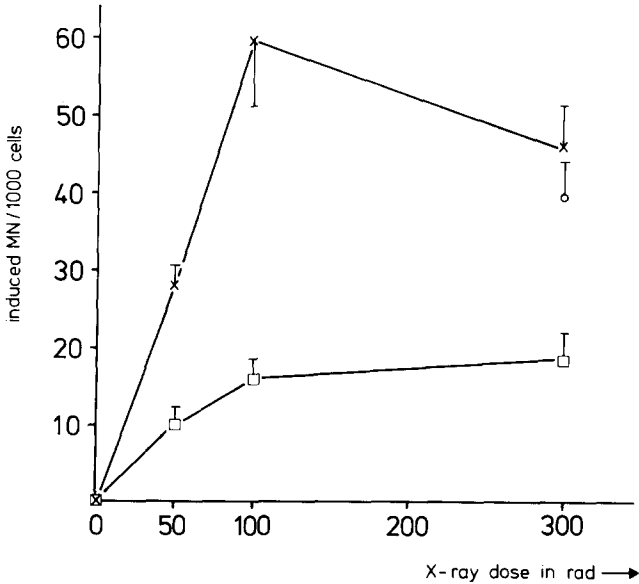


Fig. 1. Dose-response relationships for X-ray-induced MN in bone-marrow cells 18 h after irradiation.  $\times$ , Swiss mice;  $\square$ , Snell dwarf mice;  $\circ$ , Snell mice.

seemed to be absent. No change in the ratio between polychromatic erythrocytes and more mature ones was seen in normal or dwarf mice after 50 or 100 rad X-rays, indicating no strong differences in cell killing at these dose levels.

## Discussion

### *Spontaneous frequencies of MN*

In normal and dwarf Snell mice at 10 weeks of age, the spontaneous frequencies of MN were significantly higher in males than in females, a difference not found at 23 weeks of age and also not in the Swiss random-bred strain possibly owing to their age range of 10–20 weeks. Although Meyne and Legator (1980) reported higher spontaneous frequencies of MN in male Swiss mice than in females, these differences never reached statistical significance and, in all other cases studied, no differences were found between the sexes (Schmid, 1976; Chrisman and Baumgartner, 1979, 1980; Henry et al., 1980).

### *Hormone-treated dwarf mice*

Treatment of dwarf mice with growth hormone, thyroxine, insulin and testosterone did not increase the frequencies of MN present in polychromatic erythrocytes. In contrast, in the male dwarfs a significant decrease of the frequencies of spontaneous MN was found after treatment with insulin, testosterone and 1  $\mu$ g thyroxine, a dose that caused severe hyperthyroidism, whereas 0.1  $\mu$ g thyroxine, giving a euthyroid status, had an intermediate effect. This is in contrast with the results obtained by others. Judin and Antipenko (1972) found an increase in spontaneous chromosomal aberrations in rat-liver cells after thyroxine therapy. Duca-Marinescu and Negoescu (1973) found an increase in acentric fragments in bone-marrow cells of normal mice after thyroxine treatment, whereas Nilsson et al. (1975) reported an increase of the tendency for satellite-type association in lymphocytes of hyperthyroid patients. Similarly to the situation for thyroxine, Duca-Marinescu and Simionescu (1973) observed an increase for acentric fragments in mouse bone-marrow cells after treatment with growth hormone. These discrepancies between our results and others may be due to the different methods used, and more probably due to the hormone-deficient status of the dwarf mice studied here, whereas other workers used normal animals.

### *Mutagen-treated mice*

Our results on X-irradiation and mitomycin C treatment show that dwarf mice are less sensitive to induction of polychromatic erythrocytes than are normal Snell and Swiss mice. As can be expected on theoretical grounds as well as observed by Heddle (1973), Chaubey et al. (1978), Jenssen and Ramel (1978) and Jenssen (1981), the induction pattern of MN by X-rays followed with time is a wave whose shape is determined by factors such as size of dose, sensitivity of target cells, heterogeneity with respect to cell killing, cell-cycle progression and cell differentiation. The results obtained by us at different doses and different fixation times indicate a consistently

lower induction in dwarf mice suggesting no strong differences in the time sequence of cell differentiation between normal and dwarf mice.

Dose-response studies on X-ray-induced MN in bone-marrow cells have shown that, in mice, the shape is humped with a maximum around 100 rad (Chaubey et al., 1978). Our observations on Swiss mice are in line with these observations. A humped curve is generally explained in terms of coincidence of the induction of the observed parameter and cell killing in a heterogeneous population. At higher doses, where the cell-killing effect predominates, the aberration-carrying cells are selectively eliminated, resulting in a decrease of the frequencies of aberrations (Russell, 1956; Oftedal, 1968). If a stronger selection against aberration-carrying cells occurs, this will lead to lower yields of aberrations at all doses and a broadening of the peak (Lyon and Cox, 1976; van Buul, 1976 and 1980). Such a feature seems to be present in the dwarf mice. At present, it is not possible to find out whether the increased radiosensitivity with respect to cell killing of dwarf mice is due to an intrinsic situation for all cells or that it results from a difference in cell-cycle kinetics of pro-erythrocytes, leading to different proportions of cells in the various cell-cycle stages with concomitant changes in radiosensitivity. However, the observations on the ratio between the number of polychromatic erythrocytes and more mature ones present in the bone-marrow smears after treatment with X-rays or mitomycin C suggested no strong differences in cell-killing effect between dwarfs and normal mice.

Another reason for the lower frequencies of mutagen-induced MN in bone-marrow cells of dwarf mice might be a difference in repair of DNA lesions, but one can only speculate about this. Here it is just interesting to note that dwarf mice also have a lower or retarded tumour induction by several chemical carcinogens (Bielschowsky and Bielschowsky, 1959, 1960 and 1961). Our observation of no increase in MN frequency in dwarf mice after all chronic hormone treatments has to be considered in the light of these lower sensitivities of dwarfs.

The radiation experiments on dwarf mice treated with pGH plus thyroxine indicate that an increase to normal levels of MN can be obtained in these animals. This indicates that pGH plus thyroxine, at the applied concentrations, might influence the cell cycle of dwarf-mouse bone-marrow cells. It has been known for many years that different types and frequencies of chromosomal aberrations are produced by ionizing radiation at different stages of the cell cycle (Evans, 1962; Kihlman, 1977). Thus a change in the cell kinetics of pro-erythrocytes will have an effect on the radiosensitivity with respect to chromosomal aberration production.

Hormonal influence of the cell cycle has been reported by several authors. Bardik (1969) found an effect of growth hormone on the duration of the cell cycle of the oesophageal and jejunal epithelium of hypophysectomized and normal rats, whereas Romanov et al. (1969) and Romanov and Kasavina (1970) claim an increase of the mitotic activity by thyroid-stimulating hormone and thyroxine in the small intestine, bone marrow, cornea and liver of normal rats. When dwarf mice are exposed to X-rays 2 months after termination of hormone treatment, the change in radiosensitivity is still present. This suggests that the presence of the hormones at the moment of irradiation is not essential for the observed effect but that most likely irreversible changes in physiological conditions are involved. A change in the physiological



condition may very well be accompanied by a change in the metabolism of blood-forming organs by which the length of the cell cycle of mitotically dividing pro-erythrocytes can be influenced. However, the results of Guernsey et al. (1980), showing that the presence of triiodothyronine ( $T_3$ ) is necessary for neoplastic transformation by X-rays in vitro, indicate that hormones may also directly affect the radiosensitivity.

Judin and Antipenko (1972) reported a significantly lower radiosensitivity with respect to chromosomal aberration production of rat-liver cells after thyroxine treatment. This contrasts with our findings in normal Snell mice, where no change in the frequencies of radiation-induced MN was found, although these mice were treated with lower doses than the dwarfs as a result of which no increase of thyroxine level in blood was observed.

In general, it can be concluded that, in the hypopituitary dwarf mouse, growth hormone, thyroxine, insulin and testosterone do not act as mutagenic agents as judged by the MN test. The induction of MN in dwarf mice by X-rays is lower and follows a drastically different dose-effect relationship compared with that in normal mice. The lower radiosensitivity of dwarf mice can be reconstructed to normal levels by treatment with a combination of growth hormone and thyroxine, an effect that persists until at least 2 months after treatment. To interpret the mechanisms behind the induction of MN by various treatments, it is essential to gain more information on the course of events in the formation of MN in relation to different stages of the cell cycle, which can be blocked or retarded in dwarf mice.

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