Comparative Cytogenetic Studies of the Influence of Phenylbutazone and Cyclophosphamide on Spermatogenesis in the Mouse

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Abstract

In the germinal epithelia of mice (CFLP), sacrificed from one to twenty-one days after the administration of a single oral dose of phenylbutazone or cyclophosphamide, the relative frequency of spermatogonial and meiotic metaphases was determined, and the chromosomes at diakinesis-metaphase I were examined for aberrations and univalents.

After treatment with phenylbutazone (227 mg/kg), the findings were comparable with those made in the controls. In contrast cyclophosphamide (97 mg/kg) led to considerable shifts in the different types of metaphases and to a marked increase in the incidence of univalents and chromosomal aberrations; in this respect, resting spermatocytes as well as spermatogonia of type B and especially intermediate spermatogonia proved to be the most susceptible stages.

Introduction

The search for appropriate experimental models in which to assess the potential mutagenicity of chemical substances has, as yet, failed to disclose any more suitable test system than in vivo studies in mammals aimed at detecting specific effects on somatic or gonadal cells. The results of cytogenetic investigations on somatic cells, however, are not directly applicable on the germinal cells. The possible influence of drugs on the latter can be done within the scope of tests for dominant lethal effects. Such studies afford a general system that is sufficient for detecting mutagenic effects on the gametes: but an additional, comparative system, permitting chromosomal changes in the germ cells to be determined directly, may be desirable in special cases. In order to find out whether this might serve as a reliable quantitative test, a cytogenic analysis of the germinal epithelium of the male mouse was undertaken after treatment with two known drugs, phenylbutazone and cyclophosphamide.

The effect of phenylbutazone on somatic cells has already been investigated in the Chinese hamster [4] and in the dominant lethal test in the mouse [3]. These studies failed to bring to light any evidence indicating that the drug might exert mutagenic effects. Cyclophosphamide, on the other hand, is a known mutagen that can induce chromosomal alterations of varying degree in the germ cells [7].

Material and methods

(1) Animals

9-week-old inbred male mice of strain (CFLP) (Laboratory Animals Carworth-Europe, Huntingdon, England), weighing 30–40 g, were given a single oral dose of phenylbutazone (Butazolidin®, 227 mg/kg, i.e. ⅓ LD_{50}) or cyclophosphamide (Endoxan®, 97 mg/kg, i.e. ¼ LD_{50}) between 11.00 a.m. and 12.30 p.m. A control group received only the vehicle, a 0.5% solution of sodium carboxymethylcellulose. Two mice from each of the treated groups and one from the control group were killed at 11.30 a.m. on the 1st, 4th, 9th, 11th, 12th, 13th, 14th and 21st day after the administration of the substances. 3 hours prior to sacrifice, the animals were given 2 mg/kg of demecolcine (Colcemid®) intraperitoneally.

(2) Preparation of tissues

The testes were prepared according to the technique described by SCHLEIERMACHER [6]. The parenchymal tissue was immersed for 20 minutes in a hypotonic solution of 1.0% sodium citrate and 0.005% hyaluronidase (ox testis, lyophilized, ≅300 I.U./mg; FLUKA, Buchs, Switzerland) at a temperature of 37 °C. The preparations were stained with a 1% solution of aceticacid-orcein.

(3) Evaluation

The percentages of spermatogonial metaphases and metaphase I and II were determined in relation to the total number of metaphases, 300 of these divisional stages being counted per animal. In addition, diakinesis-metaphase I forms were examined as well for structural aber-

rations (Fig. 1–4) as for gonosomal and autosomal univalents (Fig. 5 and 6). Chromosomes were considered to be univalent if the distance between homologues was equal to at least the diameter of a bivalent.

The following aberrations were assessed:

Translocations (Fig. 1 and 2); *acentric fragments* (Fig. 3) and *unresolvable changes* (e.g. 'appendages', Fig. 4) were recorded.

Results

(1) Determination of the relative incidence of spermatogonial and meiotic metaphases (Table 1)

No particular deviations were observed among the control mice sacrificed at various times. The mean percentages of metaphase forms were as follows: spermatogonial metaphase (SM): 21.6; metaphase I (MI): 27.0; metaphase II (MII): 51.4. Among the mice treated with phenylbutazone only one sacrificed on the 14th day after medication showed a slight reduction in SM's.

Treatment with cyclophosphamide led to a clear-cut shift in the distribution of metaphase forms. In the animals killed on the 1st and 2nd day after medication, a slight to pronounced reduction in the percentage of SM was evident; in those killed on the 12th day, MI's accounted for only 2 and 4% of the total, while the percentage of MII forms was still relatively high (44 and 46%). In the mice sacrificed on days 13 and 14, on the other hand, the metaphase forms consisted almost exclusively of SM's. On the 21st day after medication, however, the distribution of SM: MI: MII corresponded very closely to that found in the controls.

(2) Incidence of unpaired homologous chromosomes (univalents)

The incidence of gonosomal and autosomal univalents is shown in Table 2, which also indicates the number of metaphases examined in each case and the stage of development the diakinesis-metaphase I (Dia-MI) had reached from the time of medication.

In the control group, the incidence of gonosomal univalents ranged from 0 to 4%, with an average of 1.57%; the average of the autosomal univalents was 0.42% and ranged from 0 to 1%. After treatment with phenylbutazone in the 16 mice examined (2 per stage) the mean incidence of gonosomal univalents was 2.5%, of the autosomal univalents was 0.5% in the individual stages; however, the incidence of gonosomal and autosomal univalents was never exceeding 4 and 2%, respectively.

In the mice treated with cyclophosphamide incipient changes were noted in those killed on the 9th and 11th day after medication. A considerable increase in the incidence of univalents was observed on the 12th (18% gonosomal and 6% autosomal univalents) and 13th (15% gonosomal and 11% autosomal univalents) day. In the animals killed on the 14th day, no MI's were found and univalents therefore could not be recorded. Finally, on the 21st day after medication, the incidence of univalents was again reduced (6% gonosomal univalents and 0% autosomal univalents).

(3) Analysis of chromosomes at diakinesis-metaphase I

In the control mice and in the group treated with phenylbutazone, no chromosomal aberrations were detectable at any time after medication.

The number of metaphases examined at each of the appointed times and the type and incidence of the chromosomal aberrations observed in mice treated with cyclophosphamide are shown in Table 3. No aberrations were found in the animals killed on the 1st or on the 9th day after medication, but in those killed on day 4 one unresolvable change was noted in one Dia-MI examined. On the 11th day, changes were detected in 2.5% of the cells examined. On days 12 and 13, the number of Dia-MI was considerably reduced and on the 14th day these were completely absent. The percentage of chromosomal aberrations amounted to 18 on day 12 and 20 on day 13, acentric fragments and unresolvable changes predominating. On the 21st day after medication, only one aberration – a translocation – was observed among the 100 metaphases examined.

Discussion

In these cytogenetic investigations, metaphase I was selected for the assessment of structural aberrations. Since the durations of the various phases of spermatogenesis are known [5], the changes observed can be related to the corresponding stages of development at the time of medication.

In addition, provided the duration of treatment with colchicine and hyaluronidase is held constant, conclusions concerning cytotoxic changes and, possibly, potentially mutagenic effects

Figures 1–6: Taken from the cyclophosphamide experiment.

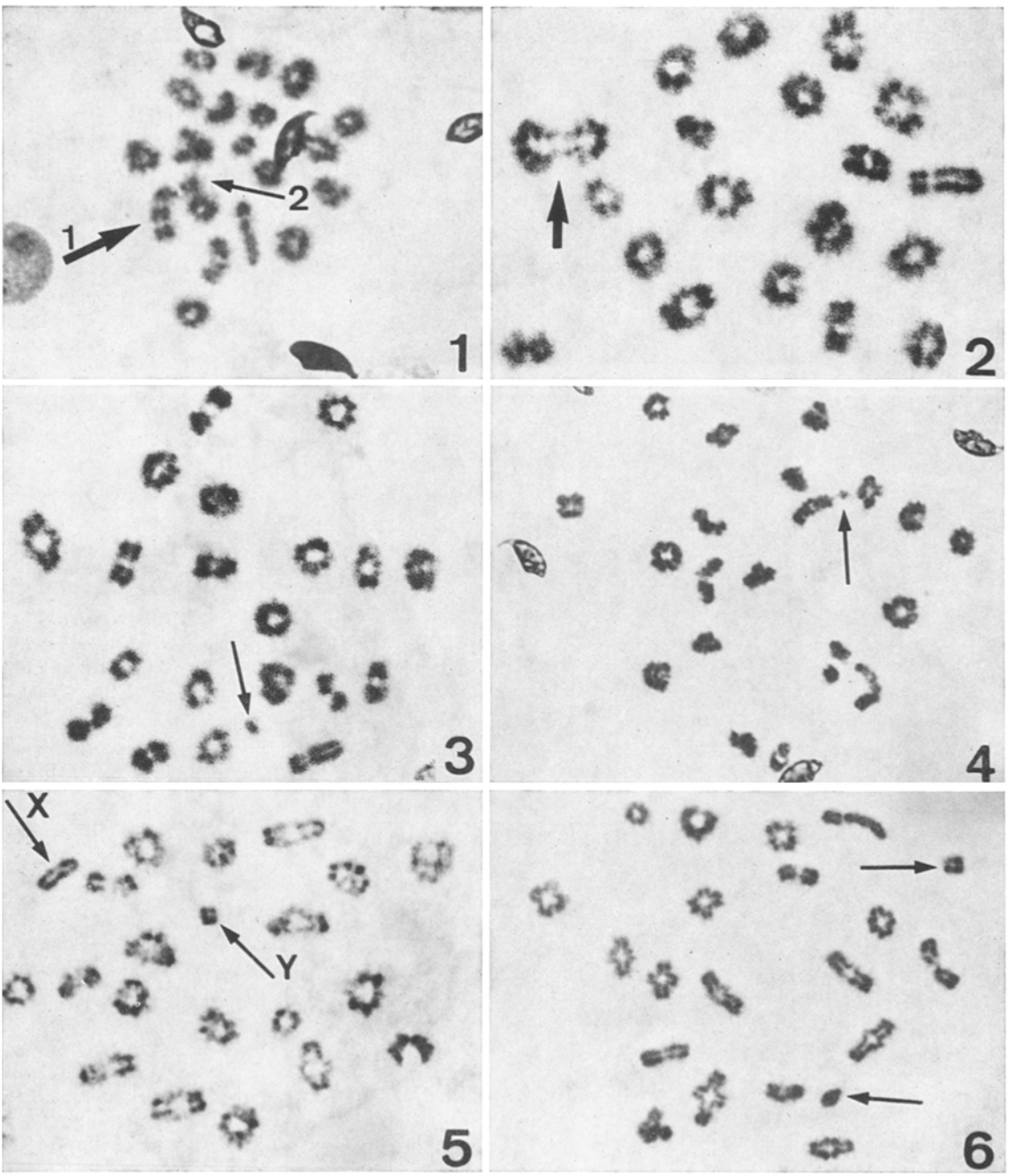

Figure 1
Treatment during Type B spermatogonial stage: arrow 1 indicates a trivalent configuration, arrow 2 indicates the univalent.

Figure 2
Treatment during leptotene; arrow indicates chain-type quadrivalent.

Figure 3
Treatment during preleptotene; arrow indicates an acentric fragment.

Figure 4
Treatment during preleptotene; arrow indicates a so called 'appendage', classified as an unresolved change.

Figure 5
Gonosomal univalent (arrows).

Figure 6
Autosomal univalent (arrows).

Table 1
Relative incidence of spermatogonial and meiotic metaphases (Spermatogonial metaphases = SM; Meiotic, metaphase I = MI, metaphase II = MII; n = 300 per percentage).

Interval between treatment and sacrifice (days)	Treatment: Controls			Cyclophosphamide (97 mg/kg)						Phenylbutazone (227 mg/kg)					
	SM	MI	MII	SM	MI	MII	SM	MI	MII	SM	MI	MII	SM	MI	MII
1	26.0	32.7	41.3	14.7	31.3	54.0	4.7	37.0	58.3	19.7	30.4	49.8	23.7	26.0	50.3
4	18.7	28.3	53.0	9.6	34.2	56.2	11.3	32.0	56.7	18.0	23.3	58.7	23.3	27.0	49.7
9	22.7	26.7	50.7	11.0	34.0	55.0	21.7	24.3	54.0	26.0	25.3	48.7	25.0	27.0	48.0
11	20.3	19.0	60.7	16.3	21.0	62.7	18.3	24.3	57.3	20.3	25.0	54.7	23.0	31.0	46.0
12	18.3	32.7	49.0	51.2	4.1	44.7	52.0	2.0	46.0	18.3	28.0	53.7	24.3	21.0	54.7
13	22.5	25.0	52.5	96.0	3.3	0.7	97.3	2.0	0.7	20.0	23.0	57.0	26.3	25.7	48.0
14	22.5	25.0	52.5	100	0	0	98.0	0	2.0	13.3	31.0	55.7	21.7	27.3	51.0
21	–	–	–	38.0	20.3	41.7	40.0	22.0	38.0	24.0	25.0	51.0	25.3	22.3	52.3

Table 2
Incidence of unpaired homologous chromosomes (univalents) in diakinesis-metaphase I.

Interval between treatment and sacrifice (days)	Stages of development at time of treatment	Number of diakinesis-metaphase I stages examined			Incidence of univalents in %					
		Controls	Phenyl-butazone	Cyclo-phos-phamide	Controls		Phenyl-butazone		Cyclophos-phamide	
					X-Y	auto-somal	X-Y	auto-somal	X-Y	auto-somal
1	Diplotene	100	100	100	1	0	3	1	2	0
4	Pachytene	100	100	100	2	0	0	2	0	1
9	Zygotene	100	100	100	0	1	4	0	2	3
11	Leptotene	100	100	200	3	1	2	0	5.5	0.5
12	Preleptotene	100	100	50	1	1	3	1	18	6
13	Spermatogonia type B	100	100	80	4	0	3	0	15	11
14	Interm. type spermatogonia	100	100	0	0	0	2	0	–	–
21	Spermatogonia type A	–	100	100	–	–	4	0	6	0

at a given stage of development can be drawn from the percentage distribution of the metaphase forms (SM, MI, MII), which is easy to determine. No attempt was made in these studies to record the absolute frequency of metaphase forms. The relative values expressed in percent are no features that can be determined independently of one another; this fact renders a definite correlation impossible. Although interphase cells could be taken as absolute reference values, their usefulness as a system of reference is limited by the fact that they may also suffer damage.

The incidence of univalent chromosomes may serve as a criterion in assessing mutagenic effects. They were found to be significantly more numerous for instance, after medication with the mutagenic alkylating agents triaziquone and cyclophosphamide [7]; and were also encountered in greater numbers after treatment with acriflavin [1].

Table 3
Chromosomal aberrations in diakinesis-metaphase I after treatment with cyclophosphamide (97 mg/kg).

Interval between treatment and examination (days)	Stage of development at time of treatment	No. of MI analysed	Number of different types of chromosomal structural changes: Translocations Trivalents (17II+III+I)	Quadrivalents (18II+CIV)	Acentric fragments	Unresolvable changes	Total number of MI with aberrations	Percentage of MI with aberrations
1	Dip	100						
4	P	100				1	1	1
9	Z	100						
11	L	200		2	2	1	5	2.5
12	R	50			3	6	9	18.0
13	B	80	2	1	7	7	17	20.0
14	In	0						
21	A	100		1			1	1

Dip = Diplotene; P = Pachytene; Z = Zygotene; L = Leptotene; R = Resting; B = Spermatogonia Type B; In = Intermediate types spermatogonia; A = Spermatogonia Type A.

In mice treated with phenylbutazone (227 mg/kg) no significant shifts in the distribution of the various metaphase forms were observed at any time after medication. The incidence of gonosomal and autosomal univalents showed no significant difference against the control, and no chromosomal changes were detectable.

Cytogenetic studies in vivo have already been carried out with phenylbutazone on somatic cells derived from the bone marrow of Chinese hamsters; in this species, oral doses of 400 mg/kg (i.e. approx. half the LD_{50}) did not give rise to any specific cytogenetic changes [4]. In a dominant lethal test carried out in mice of the CFLP strain no signs of mutagenic activity were observed after the administration of intraperitoneal doses of 100 mg/kg phenylbutazone (i.e. approx. one third of the LD_{50}) [3]. The results of the present cytogenetic investigation confirm these earlier findings.

Medication with a dose of 97 mg/kg (i.e. ¼ LD_{50}) of the active reference compound, cyclophosphamide, led to clear-cut changes in all three parameters studied. (Relative incidence of spermatogonial and meiotic metaphases, incidence of unpaired homologous chromosomes [univalents] in diakinesis-metaphase I, and number of chromosomal aberrations.)

The shifts in the metaphase ratio observed on the 1st and 4th day after administration were due to a decrease in the proportion of SM's, from which it may be inferred that the development of various spermatogonial stages was impaired. This is confirmed by the changes seen on the 11th, 12th, 13th and 14th day after medication. The proportion of MI's was reduced on the 11th day, and on the 12th, 13th and 14th day virtually none of these forms was to be found. The MII proportions followed the same pattern with a lag of one day, which corresponds to the time taken by meiotic cells to pass from MI to MII. From the durations of the various stages of spermatogenesis it may be concluded that these shifts indicate interference with the leptotene, preleptotene and Type B and intermediate spermatogonial stages. On the 12th day after medication the distribution was not yet quite normal; MII's in particular were still slightly reduced in number. This would appear to indicate that the first stages of Type A spermatogonia were also affected by cyclophosphamide.

With respect to the changes noted on the 13th and 14th day after medication, these findings are consistent with the results of similar investigations, in which interphase nuclei were taken as a reference system for MI and MII [7].

Histological studies performed in C3H mice treated with cyclophosphamide at a dose of 200 mg/kg [8] suggest that resting spermatocytes as well as Type B and especially intermediate spermatogonia are particularly susceptible. The same has also been found in the rat [3].

These observations raise the question whether it may be legitimate to infer potentially cytotoxic or mutagenic effects from shifts in the percentage distribution of the meiotic stages. In fact, the analysis of chromosomes at Dia-MI revealed aberrations coinciding with reductions in the percentage of MI. The determination of the relative percentages of SM, MI and MII forms, therefore, could serve as a general indicator of damage to the germinal epithelium.

On the 9th day after medication with cyclophosphamide, the incidence of gonosomal and autosomal univalents was 2 and 3% respectively, on the 11th day 5.5 and 0.5% respectively, i.e. they altogether were slightly more numerous than in the controls. On the 12th day they accounted for 18% gonosomal and 6% autosomal univalents, on the 13th day 15% gonosomal univalents and 11% autosomal univalents. This increase clearly coincides with the other abnormal findings, which lends support to the conclusion that a rise in the incidence of univalents may be taken as a criterion of potential mutagenicity.

Received 18 February 1972.

References

[1] K.H. BALDERMANN, G. RÖHRBORN and T.M. SCHRÖDER, *Mutagenitätsuntersuchungen mit Trypaflavin und Hexamethylentetramin am Säuger in vivo und in vitro*, Humangenetik 4, 112–126 (1967).

[2] W. HILSCHER and P. REICHELT, *Untersuchungen am Samenepithel der Ratte nach Gaben von 'Endoxan' und 'Natulan'*, Beitr. path. Anat. *137*, 452–478 (1968).

[3] L. MACHEMER and R. HESS, *Comparative Dominant Lethal Studies with Phenylbutazone, Thio-TEPA and MMS in the Mouse*, Experientia *27*, 1050-1052 (1971).

[4] D. MÜLLER and F.F. STRASSER, *Comparative Studies on the Chinese Hamster Bone Marrow after Treatment with Phenylbutazone and Cyclophosphamide*, Mutation Res. *13*, 377–382 (1971).

[5] E.F. OAKBERG, *Duration of Spermatogenesis in the Mouse*, Nature *180*, 1137–1138 (1957).

[6] E. SCHLEIERMACHER, *Über den Einfluss von Trenimon und Endoxan auf die Meiose der männlichen Maus. I. Methodik der Präparation und Analyse meiotischer Teilungen*, Humangenetik *3*, 127–133 (1966).

[7] E. SCHLEIERMACHER, *Über den Einfluss von Trenimon und Endoxan auf die Meiose der männlichen Maus. II. Cytogenetische Befunde nach Behandlung mit Trenimon und Endoxan*, Humangenetik *3*, 134–155 (1966).

[8] E. SCHLEIERMACHER, T.M. SCHROEDER, I.D. ADLER, M. VRBA and F. VOGEL, *Mutationen durch chemische Einwirkung bei Säuger und Mensch. Zytogenetische Untersuchungen in vivo und in vitro*, Dt. med. Wschr., 92. Jg., 2342–2350 (1967).