

The kinetics of nuclear polyploidization and tumour formation in livers of CF-1 mice exposed to dieldrin

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The kinetics of nuclear polyploidization in livers of CF-1 mice exposed to dieldrin were studied at concentrations of 0, 0.1, 1, 5 and 10 p.p.m. in the diet, in 'steady-state' situations (which are reached within a few weeks after initiation of treatment). Animals were killed at five time intervals (after 1.85, 3, 6, 9 and 14 months of exposure). The changes in the percentage of octaploid nuclei (8C) were used as an indicator of the kinetics of overall polyploidization. Polyploidization in control mice increased proportionally (linearly) with time (age). The enhancement of polyploidization by dieldrin was found to be proportional to dietary concentration. The slopes of the linear regressions of polyploidization, as a function of age, were identical in all dieldrin-treated groups and controls, indicating that there was no cumulative effect of dieldrin in time. A comparative analysis of the observed dieldrin dietary concentration: response relationship of polyploidization and of tumour formation in CF-1 mouse liver indicates that liver tumour formation is associated with a constant degree of polyploidization. Assuming that polyploidization reflects the ageing process, the data suggest that liver tumour formation is imminent at a constant biological age and that tumour promoters, such as dieldrin, could operate by advancing the biological age of mouse liver in the initial phases of treatment. The results of this study suggest that the analysis of ploidy changes may serve as an aid to perspective in evaluating risks associated with exposures to liver tumour promoters.

Introduction

Continuous oral exposure of CF-1 mice to dieldrin results in a sustained induction of liver microsomal enzyme systems, as well as liver enlargement associated with cellular hypertrophy and increases in total liver DNA (1–3). Within a few weeks of dieldrin treatment, the initial increases in liver size, DNA synthesis and microsomal enzyme activities are followed by a 'steady state' situation (1–3). These changes are not accompanied by evidence of liver damage, and are reversible upon withdrawal and elimination of the compound (1–3). Consequently, these phenomena are likely to be adaptive responses of liver to increased functional demands. However, prolonged exposure to dieldrin of CF-1 mice, a strain prone to 'spontaneous' liver tumour formation, has been shown to result in accelerated hepatocarcinogenesis (4–6). It would appear that the functional pressure exerted by dieldrin may facilitate the expression of intrinsic neoplastic potential in the

*Abbreviation: DAPI, 4'-6-diamidino-2-phenylindole dihydrochloride.

target organ. A similar relationship may exist between the level of functional commitments of liver cells and the kinetics of the age-associated process of nuclear polyploidization (7–9). Microsomal enzyme inducers are known to enhance, possibly accelerate, polyploidization of mouse hepatocytes (10–12). In contrast, restricted feeding or the administration of low-protein diets, which presumably diminishes the level of functional commitments in hepatocytes, have been shown to result in reduced nuclear polyploidization in mouse liver (13,14).

The objectives of the present study were (i) to elucidate the dietary dieldrin concentration: response relationship of nuclear polyploidization in livers of CF-1 mice in 'steady-state' situations; (ii) to evaluate possible similarities thereof with the dose:response relationship of enhanced mouse liver tumour formation by dieldrin, which has been reported recently (6); and (iii) to see whether (enhanced) polyploidization could serve as quantitative early marker of tumour-promoting activity in mouse liver. The kinetics of polyploidization in mouse liver parenchyma are reflected in a rise of higher ploidy classes, e.g. 8C and 16C nuclei. Accordingly, the quantitative analyses were focussed on changes in the frequency of these nuclei in response to dietary dieldrin concentration, in 'steady-state' situations. This approach excludes the interference of diploid Kupffer and endothelial cell nuclei [which may constitute up to 20% of total liver nuclei (15)] with quantitative assessments of the degree of polyploidization in liver parenchyma.

Materials and methods

Animals

CF-1 mice were kindly provided by Shell Research Ltd, Sittingbourne, Kent, UK. The colony was maintained under SPF conditions at Ivanovas GmbH, Kiesleg, FRG. Weanling female CF-1 mice were supplied to the German Cancer Research Centre upon request. The animals were allocated to groups and acclimatized for 1 week. Dieldrin treatment commenced at the age of 4–5 weeks. The animals were exposed to 0, 0.1, 1, 5 or 10 p.p.m. dieldrin in C-1000 diet (control and experimental diets were prepared by Altromin GmbH, Lage, FRG). Diet and water were given *ad libitum*. Not less than five animals/group were killed after 1.85, 3, 6, 9 and 14 months of treatment.

Isolation of liver nuclei

Animals were weighed, and killed by cervical dislocation between 9 and 10 a.m. Livers were quickly excised, the gall bladder was removed, and the tissue was chilled in ice-cold 0.25 M sucrose-TKM [0.05 M Tris-HCl, pH 7.4 (20°C), 0.025 M KCl and 0.005 M MgCl₂] for a few minutes. The livers were blotted and weighed. Liver nuclei were isolated as described by Blobel and Potter (16). Nuclear pellets were resuspended in 0.35 ml TKM buffer, and fixed by injection into tubes containing 12 ml absolute ethanol at –20°C.

Flow cytometry

DNA analysis was performed using 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI*) as the quantitative DNA fluorochrome (17). Flow cytometry was carried out with a Cytofluorograph 30 (Ortho Diagnostic Systems) connected to a computerized multichannel analyser (Plurimat, Intertechnique). The u.v. lines (351 and 364 nm) of an argon ion laser were used for DAPI excitation. DAPI fluorescence was collected through a low pass filter at 450 nm. The data obtained were displayed as frequency distributions of fluorescence intensity (a measure of nuclear DNA content). In each case 40 000 nuclei were measured. The percentages of diploid and polyploid nuclei were corrected for doublets and higher aggregates of nuclei according to Beck (18).

Table I. Percentage octaploid nuclei at different treatment intervals

Dieldrin dose (p.p.m. in diet)	Treatment		Interval		
	1.85 months	3 months	6 months	9 months	14 months
0	4.1 ± 2.0 (6) ^a	4.8 ± 1.7 (11)	6.8 ± 2.3 (11)	8.5 ± 1.9 (5)	11.5 ± 2.3 (7)
0.1	—	5.5 ± 0.7 (5)	7.3 ± 1.8 (9)	9.0 ± 2.3 (5)	—
1	—	6.2 ± 1.9 (9)	7.9 ± 1.3 (8)	9.4 ± 1.1 (5)	—
5	—	8.8 ± 2.5 (10)	10.4 ± 1.7 (7)	12.5 ± 1.5 (6) ^b	—
10	12.3 ± 2.1 (6)	12.7 ± 1.6 (9)	14.5 ± 2.9 (7)	16.1 ± 2.1 (7) ^c	—

^aNumber of animals/group is indicated in parentheses.

^bValue includes 0.7% 16C nuclei.

^cValue includes 1.9% 16C nuclei.

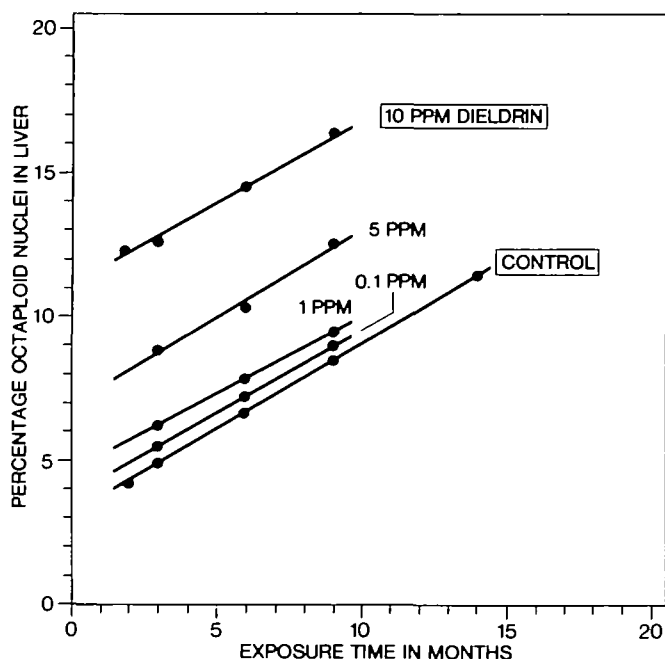


Fig. 1. Increases in octaploid liver nuclei (%) in response to dietary dieldrin concentration (p.p.m.) and exposure time.

Results

The proportion of octaploid nuclei increased linearly in all groups during the observation period of ~1 year (Table I, Figure 1). The proportion of 16C nuclei was very low (<0.3%), except in the 5 and 10 p.p.m. treatment groups at the 9-month exposure interval (0.7 and 1.9%, respectively). These results indicate that, with two exceptions, the losses of octaploid nuclei due to the formation of 16C nuclei were negligible. Accordingly, the changes in the proportion of octaploid nuclei [$\Delta(8C)$] were used as an indicator of the kinetics of polyploidization in liver parenchyma within the period of experimental observation.

The enhancement of polyploidization by dieldrin (measured in 'steady-state' situations) was found to be proportional to dietary concentration (Figure 2), and independent of the duration of dieldrin exposure. Polyploidization was analysed in untreated control CF-1 mice and found to be proportional to time (age) (Figure 3). These results show that the kinetics of polyploidization (= the changes in the proportion of octaploid nuclei) can be expressed in p.p.m. dieldrin in the diet (d) as well as in units of time (t ; Figures 2 and 3):

$$\Delta(8C) = k_1 \times d = k_2 \times t \quad (1)$$

where k_1 and k_2 are constants.

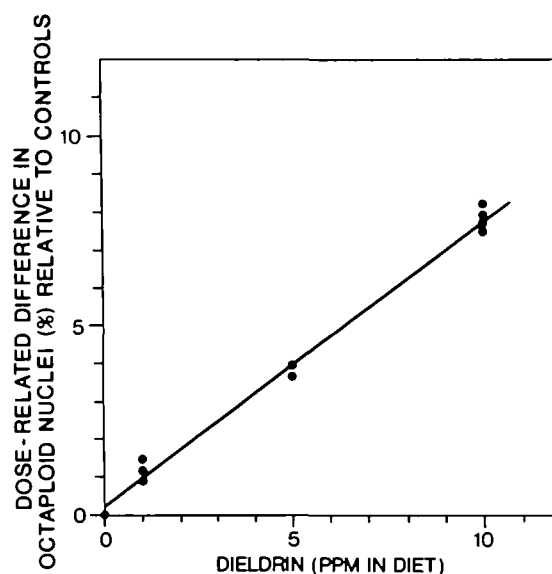


Fig. 2. Dietary dieldrin concentration-related differences (relative to controls) in octaploid liver nuclei (%) at various exposure time intervals (1.85, 3, 6 and 9 months, also see Table I).

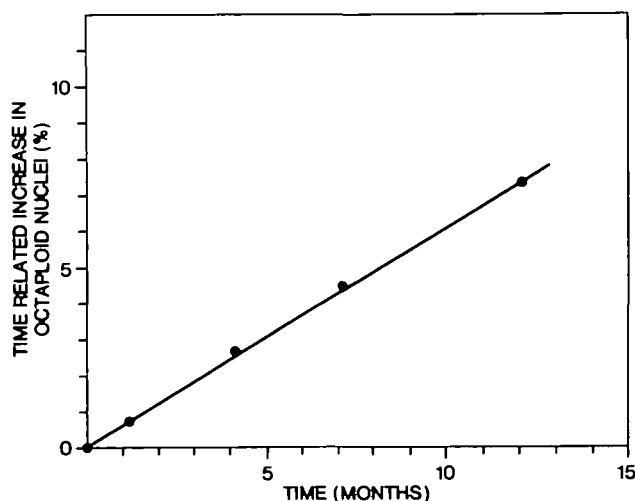


Fig. 3. Age-associated increases in octaploid liver nuclei in control mice (%). The percentage of octaploid nuclei in livers of control CF-1 mice at the 1.85 months exposure interval was used as the base line.

When polyploidization is expressed in p.p.m. dieldrin in the diet then

$$d = (k_2/k_1) \times t \quad (2)$$

This relationship is illustrated in Figure 4. The implication of

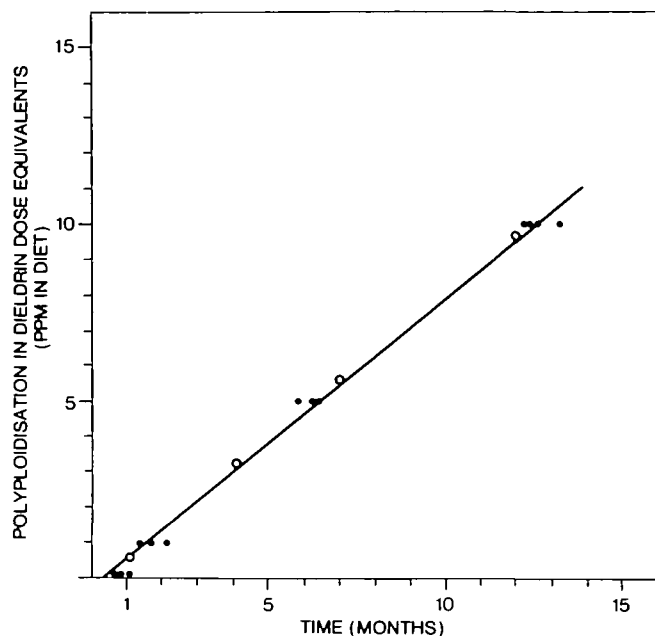


Fig. 4. Polyploidization in CF-1 mouse liver parenchyma (expressed in p.p.m. dieldrin in the diet) versus time. Age-associated increases in octaploid nuclei in control mice (O) were expressed in dietary dieldrin concentrations using the linear regression shown in Figure 2. Dieldrin-induced increases in octaploid nuclei (●) were expressed in units of time using the linear regression shown in Figure 3. Linearity leads to equation (2).

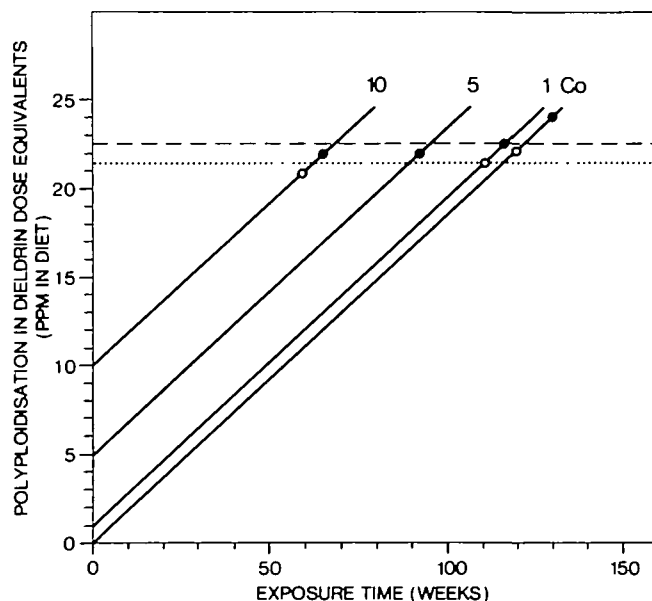


Fig. 5. Polyploidization in mouse liver parenchyma as a function of indicated dietary dieldrin concentrations and time (equation 3). Age-associated polyploidization is expressed in units of dietary dieldrin concentration (p.p.m.), using equation (2). ●, Median liver tumour induction period (= 50% incidence); ○, time to a 10% incidence of liver carcinomas.

equation (2) is that the enhancement of polyploidization by dietary dieldrin can be likened to a 'time-shift' in polyploidization associated with ageing. The extent of this time-shift which takes place in the initial phase of dieldrin treatment, i.e. before 'steady-state' is reached, is proportional to the dietary dieldrin concentration, as indicated by the parallelism of the linear regressions in Figure 1.

Age-associated polyploidization when expressed in dietary dieldrin concentrations can be viewed as the response of mouse liver to the (cumulative) action of a constant concentration of toxic substances during time (t), which would be equivalent to $d/t = 0.8$ p.p.m. dieldrin/month = 0.026 p.p.m./day, according to equation (2). Polyploidization associated with dieldrin exposure as well as with ageing ($\sim d + ct$) is illustrated in Figure 5. Ploidy status at the (known) exposure time interval associated with a cumulative frequency of 50% liver tumours (adenomas or carcinomas) or 10% liver carcinomas (6) can be estimated for each dietary dieldrin concentration and for non-dieldrin-treated controls. The results are visualised in Figure 5, and suggest a virtually constant ploidy status at the time of liver tumour (carcinoma) formation in each group.

Discussion

The enhancement of polyploidization in CF-1 mouse liver by dieldrin, analysed in 'steady-state' situations was found to be proportional to dietary dieldrin concentration, and independent of dieldrin exposure time. The absence of an 'exposure time' effect on the enhancement of polyploidization indicates that, in 'steady-state', the level of interaction of dieldrin with specific receptors does not change in the course of time. A constant and time-independent level of receptor binding implies that, in 'steady state', the velocity of association of dieldrin with specific receptors will be equal to the velocity of dissociation from these receptors (19).

Accordingly, this type of receptor binding is likely to be reversible upon withdrawal and elimination of the compound. This concept is consistent with the available evidence on the reversibility of polyploidization induced by microsomal enzyme inducers (10,12,20, B.van Ravenzwaay, unpublished results). Thus, the enhancement of polyploidization by microsomal enzyme inducers would seem to be determined by the steady state concentration at the site of interaction with specific receptors only ('Konzentrationsgift') (Table II).

Polyploidization associated with ageing, as assessed in control mice, was found to be linearly related to time. Equation (2) is consistent with the concept that age-associated polyploidization is a result of irreversible, and, therefore, cumulative interactions of toxic agents with specific receptors (Table II). Age-associated polyploidization can be regarded as a function of the product of a constant concentration of toxicants and time ('c.t.-Gift') as shown in Figure 4.

These views are consistent with the progressive nature of age-associated polyploidization. It is interesting to note, in this context, that the ploidy changes induced in rodent liver by carcinogens, such as dimethylnitrosamine, aflatoxin B₁, and 3'-methyl-4-dimethylaminoazobenzene have been reported to persist after discontinuation of treatment (21-23). This evidence may suggest similarities in the mechanisms of age-associated and carcinogen-induced ploidy changes.

Polyploidization (expressed in p.p.m. dieldrin in the diet) at the median time to liver tumour development (= 50% incidence) or at the time of a 10% carcinoma incidence was estimated for each dietary dieldrin concentration and for non-dieldrin-treated controls, and found to be virtually constant across all groups (Figure 5). At the time of a 50% liver tumour incidence there would be a level of polyploidization of 22.5 ± 0.8 p.p.m. dieldrin in the diet; at the time of a 10% incidence of liver carcinoma(s) the level of polyploidization would be 21.4 ± 0.5 p.p.m. dieldrin in the diet.

The inference that at the time of tumour formation all groups

Table II. Age- and dieldrin-related polyploidization as a function of specific receptor binding^{a,b}

Polyploidization effector	Reversibility of receptor binding	Receptor binding in relation to compound concentration	Reversibility of the effect	Effect in relation to receptor binding	Effect in relation to compound concentration	Dose-response characteristics
Dieldrin	$T_R \rightarrow 0$	$C_R \sim C$	$T_r \rightarrow 0$	$E \sim C_R$	$E \sim c$	Dose-dependent ('Konzentrations-gift')
Age(ing)	$T_R \rightarrow \infty$	$C_R \sim \int c dt$	$T_r \rightarrow 0$	$E \sim C_R$	$E \sim \int c dt$	Dose- and time-dependent ('c.t.-Gift')

^a T_R = time constant for the reversibility of receptor binding; T_r = time constant for the reversibility of the effect; c = compound concentration at the site of interaction with receptors; C_R = concentration of receptor binding; E = effect, i.e. polyploidization; t = exposure time.

^bThis concept is based on the theories developed by Druckrey and Küpfmüller (19).

would display the same degree of polyploidization suggests a strong relationship between tumour formation and polyploidization. The molecular mechanism is clearly a matter of conjecture, but some aspects can be discussed. The enhancement of polyploidization by microsomal enzyme inducers, such as dieldrin, may be triggered to meet an increased requirement for particular organ-specific activities (gene dosage), i.e. drug metabolism and SER proliferation.

If the rapid duplication of genetic material during the adaptation of the liver to increased functional demands is followed by (some) nuclear divisions (and chromosomal redistributions) in the steady-state situation, heterozygous mutations could turn homozygous as has been pointed out by Kinsella and Radman (24). In this way recessive oncogenic information could become phenotypically manifest (20).

The duplication of genetic information in polyploidization could also be triggered to save the expression of organ-specific functions from irreversible damage to functional units in the genome, a concept which has been advanced previously by Medvedev (25) and Gahan (26). This concept could be reflected by the observed age-associated increase in polyploidization.

The effect of dieldrin treatment on liver-tumour formation in CF-1 mice can be viewed as a 'time-shift', in 'spontaneous' tumour formation (6).

A similar 'time-shift' is apparent for the enhancement of polyploidization by dieldrin (Figures 2 and 4). Judged by ploidy status, dieldrin appears to create a dose-dependent 'time-gap' between chronological and biological age of mouse liver in the initial phase of treatment. The data are consistent with the view that tumour formation is imminent at a constant biological age of mouse liver and that tumour promoters may operate by advancing the biological age of their target organ in the initial phases of treatment. Thus, the close similarities in the kinetics of polyploidization and tumour formation in livers of CF-1 mice exposed to dieldrin suggest that the analysis of ploidy status may serve as an aid to perspective in assessing risks posed by exposure to liver-tumour promoters.

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