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Genetic Effects of Dioxins in the Spot Test with Mice

by Rudolf Fahrig

More than any other environmental chemicals, dioxins have been in the limelight of public interest for about 10 years. In addition to carcinogenicity, genetic risk is a cause for concern. Mutagenicity tests performed so far do not give a clear picture. The mutagenic potential of dioxins has to be considered weak or absent. Therefore, it seemed profitable to investigate comutagenicity and co-recombinogenicity of dioxins more thoroughly. The only useful method for investigating comutagenicity and co-recombinogenicity of dioxins *in vivo* is the spot test with mice. In this test system, a number of cocarcinogens and tumor promoters have shown comutagenic or co-recombinogenic effects. In the present study, tetrachlorodibenzo-*p*-dioxin (TCDD) and two environmental dioxin mixtures [pentachlorodibenzodioxin (PCDD) 1 and 2] were tested for genetic activity. Given alone, no mutagenic or recombinogenic effects could be observed. In combination with the carcinogenic mutagen ethyl nitrosourea (ENU) at concentrations of 128 μg/kg for PCCD 2, 314 μg/kg for PCDD 1, and 3 μg/kg for TCDD, a doubling of the genetic effectiveness of ENU was observed. The genetic risk can roughly be considered as 1:0.02 for TCDD:PCDD 2 and 1:0.01 for TCDD:PCDD 1. While PCDD 1 and 2 seem to enhance the mutagenic as well as the recombinogenic potential of ENU, TCDD showed mainly co-recombinogenic and antimutagenic activity. This characteristic indicates that TCDD is mainly a tumor promoter.

Introduction

The aim of this study was to estimate the genetic risk of two environmental dioxin mixtures in comparison to 2,3,7,8-tetradichlorodibenzo-*p*-dioxin (TCDD). It is known that TCDD is carcinogenic (*1–5*), but it is nearly ineffective in genotoxicity tests (*6–12*). Therefore, it did not seem useful to perform another test for mutagenicity. Instead, I examined comutagenic and co-recombinogenic effects. The reason for doing this was *a*) TCDD has shown cocarcinogenic and tumor-promoting activities (*13–15*) and *b*) in yeast and mice, cocarcinogens have shown comutagenic effects and tumor promoters have shown co-recombinogenic effects (*16–18*). The only useful method for the present study seemed to be the spot test with mice (*19*) because in this test system a number of cocarcinogens and tumor promoters have shown comutagenic or co-recombinogenic effects (*16,17*). The effect of tumor promoters of the phorbol ester type could be measured at concentrations as low as 30 μg/kg. Therefore, the sensitivity of the method includes toxicologically relevant doses of dioxins.

The spot test was introduced in 1975 for studies of genotoxicity (*19*). The exceptional feature of the spot test is that it allows one to distinguish between induced gene mutations and induced reciprocal recombinations (*16*).

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Table 1.[a]

Congeners	PCDD mixture 1, mg	PCDD mixture 2, mg
Tox-equivalency (BGA)	2.84	6.29
sum TCDD	4.64	24.03
sum PeCDD	26.48	76.42
sum HxCDD	52.54	136.63
sum HpCDD	114.23	151.74
sum OCDD	239.39	57.53
sum PCDDs	437.29	446.35
TCDD/PCDD (%)	1.06	5.38
PeCDD/PCDD, %	6.05	17.12
HxCDD/PCDD, %	12.02	30.61
HpCDD/PCDD, %	26.12	34.00
OCDD/PCDD, %	54.75	12.89

Abbreviation: BGA, Bundesgesundheitsamt (Federal Health Office).
[a]Producer: mixture I + II: Institut für Organische Chemie, Universität Tübingen.

Material and Methods

TCDD of > 98% purity was obtained from Radian Corporation (Woburn, MA). Ethylnitrosourea (ENU) was obtained from Ferak (Berlin, Germany). The dioxin mixtures used are shown in Table 1.

Spot Test

According to the spot test, mouse embryos, which are heterozygous for different recessive coat color genes, are treated *in utero* between 9 and 11 days after conception by injection of a mutagen into the peritoneal cavity of the

Table 2. Theoretical expectations.

	Original state					
	b/B	*p/P*	*d/D*	c^{ch}/C		$p\ c^{ch}/P\ C$
Color spots induced by						
Mutation (*a*)	b/b^a	p/p^a	d/d^a	c^{ch}/c^a	–	–	–
Deletion (Δ)	*b*/Δ	*p*/Δ	*d*/Δ	c^{ch}/Δ	($p\ c^{ch}/\Delta$)	–	(*P C*/Δ)
Monosomy (*o*)	*b/o*	–	*d/o*	–	($p\ c^{ch}/o$)	–	(*P C/o*)
Reciprocal recombination	*b/b*	*p/p*	*d/d*	–	$p\ c^{ch}/p\ c^{ch}$	Twin spot	*P C/P C*
Nonreciprocal recombination (*)	*b/b**	*p/p**	*d/d**	–	–	–	–
Color of spot	Brown	Light gray	Gray	Light brown	Near-white		Maternal black

mother animal or by other appropriate routes of administration. If this treatment leads to an alteration or loss of a specific wild-type allele in a pigment precursor cell, a color spot in the coat of the adult animal may appear.

With regard to the mechanism (Table 2) by which the heterozygous recessive coat-color alleles can be expressed, this is either a gene mutation, theoretically also loss of the wild-type allele through deletion or monosomy, a recombinational process such as mitotic crossing-over (reciprocal recombination), or mitotic gene conversion (nonreciprocal recombination). Of the numerical and structural chromosome aberrations that can lead to loss of the wild-type allele, only those that survive several mitoses would cause a spot with expression of the recessive allele. In the routinely performed spot test, three types of spots are distinguished: *a*) white midventral spots (which have no pigment at all). These are regarded as resulting from pigment cell killing; *b*) spots with hairs similar to the yellow hairs which normally surround ears, genital papillae, and mammae. These are classified as misdifferentiation spots and appear as yellow fluorescent hairs with agouti genotype; *c*) spots of genetic relevance (SGR) resulting from genetic alterations at the different gene loci and expressing the recessive mutant or their wild-type alleles.

Without routinely performed microscopical analysis, it is not possible to distinguish between the different mechanisms leading to expression of a recessive mutation. The only possibility to distinguish between induced mutations and induced reciprocal recombinations is to use specific mouse strains and to identify the gene loci involved in appearance of a color spot by microscopical pigment analysis (*16,20*).

The embryos treated were the F_1 from the cross C57Bl × T, being homozygous for nonagouti (*a/a*), and heterozygous for brown (*b/B*), pink-eyed dilution and chinchilla ($p\ c^{ch}/P\ C$), dilute and short ear (*d se/D SE*), and piebald spotting (*s/S*). Mutations of piebald spotting or short ear cannot be detected using the spot test. Heterozygosity of the recessive mutant alleles leads to dark gray coat in the F_1. In contrast, the mother animal homozygous for the wild-type alleles has a black coat.

Gene mutations can now be detected as genetic alterations at the *c* locus; c^{ch}/c^{ch} in combination with *a/a* results in a dull black or sepia color spot, neither of which contrasts clearly from the coat. However, the genetic alteration that can be detected is a mutation of *c* or a lethal allele of *c*, both of which combined with c^{ch} give rise to a light brown c/c^{ch} phenotype. Therefore, light brown spots are caused only by gene mutation or small deletions, but not by recombinations.

It is possible to detect reciprocal recombinations between the *p* and *c* loci because the loci are located on the same chromosome (14 units apart). A genetic alteration leading to $p\ c^{ch}/p\ c^{ch}$ or $p\ c^{ch}/\Delta$ (Δ = deletion) gives rise to near-white color spots, the characteristic reduction in pigmentation being clearly identifiable by microscopical analysis. Near-white spots are unlikely to be due to gene mutations because simultaneous mutations at the linked loci *p* and *c* are extremely rare and have never been observed in specific-locus experiments. It is also highly unlikely that large deletions involving both the *c* and *p* loci are sufficiently viable in the heterozygous form or in the case of monosomy. The most likely genetic alteration leading to viable cells of the genotype $p\ c^{ch}/p\ c^{ch}$ is reciprocal recombination due to mitotic crossing-over. The corresponding reciprocal product of mitotic crossing-over is cells of the genotype P C/P C. The detection of P C/P C is possible because the recessive genes, even in the heterozygous state, have an influence on the level of pigmentation. In contrast to the homozygous nonagouti black mother animals, F_1 animals are dark gray to black on the back, and medium gray on the ventral side. Therefore, pigment cells of the genotype P C/P C show up as black spots. Color pictures of spots and hair pigment have been published recently (*20*).

A feature of mitotic crossing-over is the potentiality for forming twin spots. A twin spot, homozygous for the recessive markers and their wild-type alleles, respectively, are both distinguishable from the heterozygous remainder of the body. It is not necessary that both spots should be visible; the descendants of either of the daughter cells may not occupy a position on the surface, or where the marker gene can express itself. Therefore, the appearance of twin spots is a rare event.

Results

The results summarized in Table 3 and Figure 1 clearly show that the dioxin mixtures as well as TCDD enhance the genotoxicity of ENU. A doubling of the effect of ENU can be observed at different concentrations: 314 μg/kg PCDD2, 128 μg/kg PCDD1, and 3 μg/kg TCDD. Given alone, 128 μg/kg PCDD2 was ineffective in inducing mutations or recombinations.

Table 3. Effect of dioxins in the spot test.

Substance	Dose, μg/kg	Females with vaginal plug: Day after conception	Females with vaginal plug: No. treated	Females with vaginal plug: No. with litter	F_1-animals 4 weeks old	F_1-animals midventral white	F_1-animals with color spots of genetic relevance
PCDD II + ENU	1021	9	79	16	10	2	2
ENU	30,000	9	35	13	57	1	3[a]
PCDD II + ENU	128	9	109	34	151	13	28[b]
ENU	30,000	9	61	25	107	8	12
PCDD I + ENU	314	9	84	28	141	25	27
ENU	30,000	9	39	17	98	5	11
PCDD II	128	9	120	38	168	0	1
DMSO		9	120	43	208	1	3
TCDD + ENU	3	9	100	49	178	32	37[c]
ENU	30,000	9	100	41	223	11	19[d]
TCDD + ENU	3	9	100	44	198	30	39[d]
ENU	30,000	9	100	42	216	19	27

[a]One animal with two spots of different colors.
[b]Three animals with two spots of different colors.
[c]Four animals with two spots of different colors.
[d]Two animals with two spots of different colors.

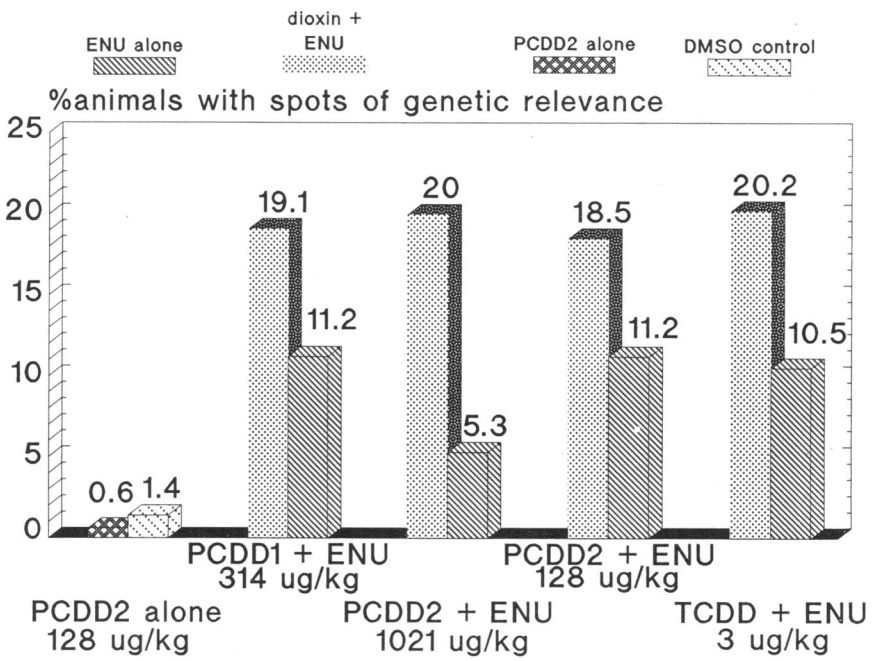

FIGURE 1. Enhancement of the genetic effects of ethylnitrosourea by treatment with dioxins.

The results summarized in Table 4 and Figures 2 and 3 allow detection of comutagenic or co-recombinogenic effects. Considering spots that could have been induced by both, recombinations and mutations, no influence of dioxins can be observed. The two dioxin mixtures do not seem to be able to enhance specifically either the mutagenic or recombinogenic effect of ENU. In contrast to this, TCDD shows a clear co-recombinogenic and antimutagenic effectiveness. As can be seen in Table 3 and Figure 3, the frequency of twin spots induced by ENU and TCDD is 3.7%. With ENU alone, only 2 (0.2%) of 858 color spots were twin spots (*21*). Within the present positive controls, with ENU alone no twin spot could be induced. A clear antimutagenic effect of TCDD is apparent when comparing the frequency of light brown spots (Table 3 and Figures 2 and 3). Summarizing, it can be said that the

Table 4. Distribution of color spots among four gene loci in the mammalian spot test.

	Original state						
				c^{ch}/C		$pc^{ch}PC$	
	b/B, brown	*p/P*, light gray	*d/D*, gray	Light brown	Near-white	Twin spot	Maternal black
2 spots induced with 1021 μg/kg PCDD II + ENU were:	–	1	1	–	–	–	–
4 spots induced with ENU alone were:	–	2	2	–	–	–	–
31 spots induced with 128 μg/kg PCDD II + ENU were:	7	10	10	3	1	–	–
12 spots induced with ENU alone were:	3	7	1	–	1	–	–
27 spots induced with 314 μg/kg PCDD I + ENU were:	2	9	10	3	2	–	1
11 spots induced with ENU alone were:	1	6	2	1	1	–	–
1 spot after treatment with 128 μg/kg PCDD II was:	–	–	1	–	–	–	–
3 spots after treatment with 0.01 mL/10 g DMSO were:	2	–	1	–	–	–	–
3 μg/kg TCDD + 30 mg/kg ENU							
Experiment 1 (41 color spots)[a]	4	16	14	1	5	–	1
Experiment 2 (41 color spots)[a]	4	8	19	3	4	3	–
30 mg/kg ENU							
Experiment 1 (20 color spots)[a]	1	7	8	3	–	–	1
Experiment 2 (27 color spots)[a]	6	6	8	4	3	–	–

[a]Treatment of 100 mother animals.

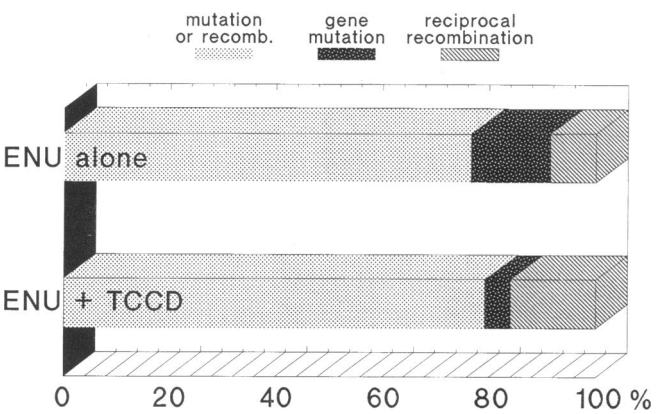

FIGURE 2. Percentage of color spots induced either by gene mutation or by recombination.

dioxin mixtures enhance the genetic effectiveness of ENU in an unspecific way, whereas TCDD shows clear co-recombinogenic and antimutagenic effects.

Discussion

The aim of the study was to compare the genetic risk of two environmental dioxin mixtures with TCDD. In toxicology this is done by introducing equivalency factors using TCDD = 1 as a reference quantity. Normally, the criteria for estimation of equivalency factors have nothing to do with toxicology. Instead, binding affinity and induction of enzymes are used. The results of such calculations are insufficient for any form of risk estimation, and especially for estimation of genetic risks. The present work may be more useful in this respect. As 3 μg/kg TCDD are as effective as 128 μg/kg PCDD2 mixture or 314 μg/kg PCDD1 mixture, the genetic risk of TCDD:PCDD2 is about 1:0.02, and that of TCDD:PCDD1 about 1:0.01.

In contrast to the two dioxin mixtures, TCDD showed a specific co-recombinogenic and antimutagenic effect. Such specific effects have been observed before for several tumor promoters (*16–18*).

The relationships between the effects of substances in carcinogenicity tests and in genetic experiments do not prove that there is a causal connection between the two processes, but they offer at least plausible explanations for hitherto conflicting results in carcinogenicity experiments. A simple desmutagenic effect in the genetic experiments can be excluded because of the parallel enhancement of recombinations. Thus, the genetic effects observed may be relevant to the carcinogenic process.

If initiation is based on mutation, it seems plausible that cocarcinogens may act as comutagens. But the question arises of why tumor promoters promote induction of recombinations rather than mutations. A possible explanation comes from experiments using yeast, in which the probability that a heterozygous recessive gene becomes homozygous is two orders of magnitude higher for nonreciprocal recombination than for gene mutation (*22,23*). Also, observations in cultured mouse cells showed that the frequency of nonreciprocal recombination (gene conversion) between repetitive genes is several orders of magni-

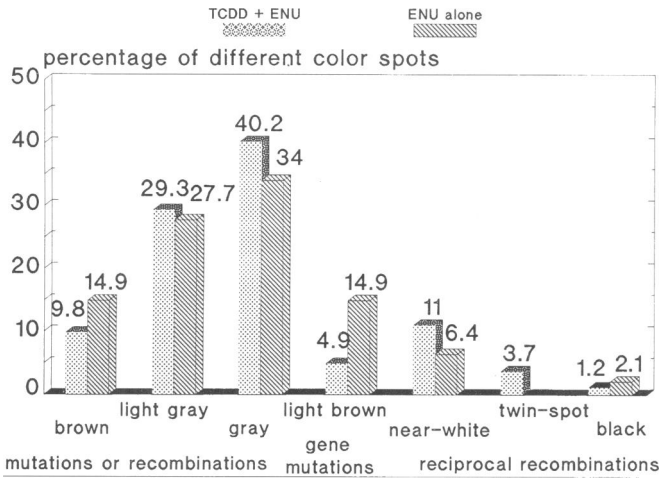

FIGURE 3. Color spots induced with 3 μg/kg TCDD plus 30 mg/kg ethylnitrosourea distributed to different gene loci.

tude higher than the frequency of gene mutation (*24*). With reciprocal recombination, a single event is sufficient to result in the expression of all recessive mutations of a chromosomal segment, whereas with gene mutations several single events would be needed to achieve a similar effect. Thus, if a tumor promoter would channel the spontaneously occurring genetic alterations into the pathway of recombination rather than mutation, the chance of recessive tumor genes (induced by an initiator) being expressed would be increased. In any case, the co-recombinogenic effects observed are useful for estimation of the genetic risk. It is possible to distinguish between TCDD and other dioxins in respect to the nature of their genetic effectiveness and the strength of this effect.

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