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 [pentachlorodibenzo-dioxin (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 PCDD 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 100% for TCDD:PCDD 2 and 100% 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.

Table 1.

| Congeners     | PCDD mixture 1 | PCDD mixture 2 |
|---------------|----------------|----------------|
|               | mg             | 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).

*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.

| Color spots induced by                 | b/B | p/P | d/D | c/h/C | p c/h/P C |
|----------------------------------------|-----|-----|-----|-------|-----------|
| Mutation (a)                           | b/b*| p/p*| d/d*| c/h/c*| —         |
| Deletion (Δ)                           | b/Δ | p/Δ | d/Δ | c/h/Δ | (p c/h/Δ) |
| Monosomy (o)                           | b/o | —   | —   | —     | (p c/o)   |
| Reciprocal recombination               | b/b | p/p | d/d | —     | Twin spot |
| Nonreciprocal recombination (*)        | b/b*| p/p*| d/d*| —     | —         |
| Color of spot                          | Brown | Light gray | Gray | Light brown | Near-white | Maternal black |

R. FAHRIG

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 recombination 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 mammea. 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 recombination 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 F1 from the cross C57Bl × T, being homozygous for nonagouti (a/a), and heterozygous for brown (b/B), pink-eyed dilution and chinchilla (p c/h/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 F1. 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/h/c* in combination with a/a results in a dull black or sephia 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*h give rise to a light brown c/c*h phenotype. Therefore, light brown spots are caused only by gene mutation or small deletions, but not by recombinations.

It is possible to detect reciprocal recombination 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/h/p c/h or p c/h/Δ (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/h/p c/h 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, F1 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 | Day after conception | No. treated | No. with vaginal plug | F₁-animals with color spots of genetic relevance |
|---------------|-------------|----------------------|-------------|-----------------------|--------------------------------------------------|
| PCDD II + ENU | 1021        | 9                    | 79          | 16                    | 2, 2                                              |
| ENU           | 30,000      | 9                    | 35          | 13                    | 1, 3                                              |
| PCDD II + ENU | 128         | 9                    | 109         | 34                    | 13, 28b                                           |
| ENU           | 30,000      | 9                    | 61          | 25                    | 8, 12                                             |
| PCDD I + ENU  | 314         | 9                    | 84          | 28                    | 25, 11                                            |
| ENU           | 30,000      | 9                    | 39          | 17                    | 5, 11                                             |
| PCDD II       | 128         | 9                    | 120         | 38                    | 1, 1                                              |
| DMSO          |             |                      |             |                       | 0, 1                                              |
| TCDD + ENU    | 3           | 9                    | 100         | 49                    | 32, 37c                                           |
| ENU           | 30,000      | 9                    | 100         | 41                    | 11, 19d                                           |
| TCDD + ENU    | 3           | 9                    | 100         | 44                    | 30, 39d                                           |
| ENU           | 30,000      | 9                    | 100         | 42                    | 19, 27                                            |

*One animal with two spots of different colors.
*bThree animals with two spots of different colors.
*cFour animals with two spots of different colors.
*dTwo animals with two spots of different colors.

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 anti-mutagenic 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 558 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 | 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)* | 4 | 16 | 14 | 1 | 5 | – | 1 |
| Experiment 2 (41 color spots)* | 4 | 8 | 19 | 4 | 3 | – | 3 |
| 30 mg/kg ENU |  |  |  |  |  |  |  |
| Experiment 1 (20 color spots)* | 1 | 7 | 8 | 3 | – | – | 1 |
| Experiment 2 (27 color spots)* | 6 | 6 | 8 | 4 | 3 | – | 1 |

*Treatment of 100 mother animals.

Dioxin mixtures enhance the genetic effectiveness of ENU in an unspecified 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 non-reciprocal 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-
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 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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REFERENCES

1. Kochba, R. J., Keyes, D. G., Beyer, J. E., Carreon, R. M., Wade, C. E., Dittember, A., Kainins, R. P., Frauson, L. E., Park, C. N., Barnard, S. D., Hummel, R. A., and Humiston, C. G. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. Toxicol. Appl. Pharmacol. 46: 279–303 (1978).

2. Van Miller, J. P., Lalich, J. J., and Allen, J. R. Increased incidence of neoplasm in rats exposed to low levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Chemosphere 6: 537–544 (1977).

3. NTP. Carcinogenesis Bioassay of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin in Osborne–Mendel Rats and B6C3F1 Mice (gavage study). Technical Report Series No. 209, Toxicology Program, Research Triangle Park, NC, 1982.

4. IARC. Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Vol. 15. Some Fumigants, the Herbicides 2,4-D and 2,4,5-T, Chlorinated Dibenzo- and Miscellaneous Industrial Chemicals. International Agency for Research on Cancer, Lyon, 1977, pp. 41–102.

5. NCI/NTP Carcinogenesis Bioassay of 2,3,7,8-Dichlorodibenzo-p-Dioxin (TCDD) for Possible Carcinogenicity. Technical Report Series No. 123, National Cancer Institute/National Toxicology Program, Bethesda, MD, 1979.

6. Jackson, W. T. Regulation of mitosis. III. Cytological effects of 2,4,5-trichlorophenoxyacetic acid and of dioxin contaminants in 2,4,5-T formulations. J. Cell Sci. 10:15–25 (1972).

7. Wassom, J. S., Huff, J. E., and Loprieno, N. A review of the genetic toxicology of chlorinated dibenzo-p-dioxins. Mutat. Res. 47: 141–160 (1977).

8. Geiger, L. E., and Neal, R. A. Mutagenicity testing of 2,3,7,8-tetrachlorodibenzo-p-dioxin in histidine auxotrophs of Salmonella typhimurium. Toxicol. Appl. Pharmacol. 59: 125–129 (1981).

9. Rogers, A. M., Andersen, M. E., and Back, K. C. Mutagenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin and perfluoro-n-decanolic acid in L5178Y mouse lymphoma cells. Mutat. Res. 105: 445–449 (1982).

10. Meyne, J., Allison, D. C., Bose, K., Jordan, S. W., Didolopho, P. F., and Smith, J. Hepatotoxic doses of dioxin do not damage mouse bone marrow chromosones. Mutat. Res. 157: 63–69, (1985).

11. Mortelmans, K., Haworth, S., Speck, W., and Zeiger, E. Mutagenicity testing of agent orange components and related chemicals. Toxicol. Appl. Pharmacol. 75: 137–146 (1984).

12. Giri, A. K. Mutagenic and genotoxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a review. Mutat. Res. 168: 241–248 (1986).

13. Kouri, R. E., Rude, T. H., Joglekar, R., Dansette, P. M., Jerina, D. M., Atlas, S. A., Owens, I. S., and Nebert, D. W. 2,3,7,8-Tetrachlorodibenzo-p-dioxin as cocarcinogen causing 3-methylcholanthrene-initiated subcutaneous tumors in mice genetically “nonresponsive” at an locus. Cancer Res. 38: 2777–2783 (1978).

14. Pitot, H. C., Goldsworthy, T., Campbell, H. A., and Poland, A. Quantitative evaluation of the promotion by 2,3,7,8-tetrachlorodibenzo-p-dioxin of hepatocarcinogenesis from diethylnitrosamine. Cancer Res. 40: 3616–3620 (1980).

15. Poland, A., Pafen, D., and Glover, E. Tumor promotion by TCDD in skin of HRS/J hairless mice. Nature 300: 271–273 (1982).

16. Fahrig, R. Genetic mode of action of cocarcinogens and tumor promoters in yeast and mice. Mol. Genet. Genet. 194: 7–14 (1984).

17. Fahrig, R. Enhancement of carcinogen-induced mutations or recombinations by 12-O-tetradecanoylphorbol-13-acetate (TPA) in the mammalian spot test J. Cancer Res. Clin. Oncol. 113: 61–66 (1987).

18. Fahrig, R. Effects of bile acids on the mutagenicity and recombinogenicity of triethylene melamine in yeast strain MPI and D61. M. Arch. Toxicol. 60: 192–197 (1987).

19. Fahrig, R. A mammalian spot test: Induction of genetic alterations in pigment cells of mouse embryos by X-rays and chemical mutagens. Mol. Genet. Genet. 138: 309–314 (1975).

20. Fahrig, R., and Neuhäuser-Klaus, A. Similar pigmentation characteristics in the specific locus and the mammalian spot-test. J. Hered. 76: 421–426 (1985).

21. Fahrig, R. Tests for recombiningens in mammals in vivo. Mutat. Res. 284: 177–183 (1992).

22. Fahrig, R. Evidence that induction and suppression of mutations and recombination by chemical mutagens in S. cerevisiae during mitosis are jointly correlated. Mol. Genet. Genet. 169: 125–139 (1979).

23. Fahrig, R. The effect of dose and time on the induction of genetic alterations in Saccharomyces cerevisiae by aminoacridines in the presence and absence of visible light irradiation in comparison with the dose-effect-curves of mutagens with other type of action. Mol. Genet. Genet. 144: 151–140 (1976).

24. Lisjak, R. M., and Starchek, J. L. Evidence for intrachromosomal gene conversion in cultured mouse cells. Cell 33: 157–165 (1983).