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Quantitative Evaluation of the Promotion by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin of Hepatocarcinogenesis from Diethylnitrosamine[1]

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ABSTRACT

In order to test the potential of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as a promoter of hepatocarcinogenesis, rats which had received a single 10-mg/kg dose of diethylnitrosamine (DEN) following partial hepatectomy were given TCDD (0.14 and 1.4 μg/kg s.c. once every 2 weeks) for 7 months. Animals which received (*a*) only a single initiating dose of DEN after partial hepatectomy and no further treatment of (*b*) TCDD alone with no initiating dose of DEN exhibited relatively few enzyme-altered foci and no hepatocellular carcinomas. However, animals initiated with DEN and then given TCDD had a marked increase in enzyme-altered foci. At the higher dose of TCDD, hepatocellular carcinomas were present in five of seven rats. By means of three different enzyme markers used to evaluate the phenotypes of the enzyme-altered foci, a distinct phenotype heterogeneity of the foci was noted with a shift towards phenotypes exhibiting a greater deviation from normal liver when TCDD was given following DEN-partial hepatectomy. Quantitation of the numbers of enzyme-altered foci was performed by relating measurements made from two-dimensional tissue sections to the numbers of foci per unit volume of liver using relationships established in the field of stereology. The total volume of the liver occupied by the enzyme-altered foci, but not their number, increased with the dose of TCDD administered following DEN-partial hepatectomy. These studies demonstrate that TCDD is a potent promoting agent for hepatocarcinogenesis.

INTRODUCTION

TCDD[4], a trace contaminant formed in the commercial synthesis of the herbicide, 2,4,5-trichlorophenoxyacetic acid, is an extraordinarily potent toxin and teratogen (33). TCDD is the prototype of a large series of halogenated aromatic hydrocarbons including dibenzo-*p*-dioxins, dibenzofurans, azo- and azoxybenzenes, biphenyls, and naphthalenes which are all approximate isostereomers, produce similar biochemical actions, and produce a similar and characteristic pattern of toxic responses (10, 26). These compounds are all thought to exert their toxic action by a common mechanism (24).

Recently, TCDD has been shown to be carcinogenic in chronic feeding experiments in rats and mice (15).[5] Kociba *et al.* (15) reported that a dietary intake of 0.1 μg/kg/day for 2 years resulted in an increased number of hepatocellular carcinomas and squamous cell carcinomas of the lung, hard palate, and nasal turbinate in female Sprague-Dawley rats. Lifetime feeding of TCDD equivalent to 0.001 and 0.01 μg/kg/day produced no increase in tumor incidence in rats. At a daily dose of 0.1 μg/kg/day, TCDD produces nearly a 50% incidence in female rats of one of the 3 cancers listed above, making it one of the most potent carcinogenic agents known (23).

The carcinogenic potency of TCDD is especially interesting in light of studies which have failed to demonstrate any covalent binding of TCDD (23, 29, 40) and have provided inconclusive evidence that TCDD is a weak mutagen (41). Following the *in vivo* administration of [^{3}H]TCDD to Sprague-Dawley rats, the radioactivity associated with purified DNA from liver, a maximal estimate of covalent binding, was 6 pmol of TCDD per mol of nucleotide (23). This is 4 to 6 orders of magnitude lower than the binding observed with most chemical carcinogens. It seems unlikely that TCDD-induced oncogenesis is mediated through the covalent binding of this compound to DNA and subsequent somatic mutation.

Tumor development in the skin has been shown to occur in 2 stages, initiation and promotion (2, 3, 18, 35). Initiation is an irreversible process, which results from the administration of a carcinogen, and it presumably involves the covalent binding of the carcinogen or an active metabolite to DNA (20). Promotion is a reversible process, influenced by many factors (3, 21, 39), which converts the irreversibly altered, "initiated" cell into a neoplasm. Studies by Peraino *et al.* (19) have demonstrated a 2-stage model of carcinogenesis in the liver by the phenobarbital promotion of acetylaminofluorene-initiated hepatic cells. These studies have been confirmed by other investigators (14, 42) and have extended our knowledge of tumor promotion (13).

Since TCDD does not appear to be an initiator (*i.e.*, there is no conclusive evidence that it is a mutagen), we considered the hypothesis that the liver cancer associated with chronic administration of TCDD might arise from the promoting activity of the compound, presumably stimulating cells already spontaneously initiated by dietary and other environmental carcinogens. To study this question, we used a 2-stage model of hepatocarcinogenesis developed in our laboratory (21, 34) based on studies of Peraino, Scherer, Emmelot, and others (1, 8, 19, 28, 31). Rats were subjected to a partial hepatectomy to stimulate cell division, and 24 hr later received a single low dose of DEN by intubation. The animals were then treated by chronic administration of a promoting agent, *i.e.*, phenobarbital, beginning 1 to 2 months later. After a single low dose of DEN, we demonstrated that chronic dietary administration of phenobarbital resulted in the hepatocellular carcinomas and a marked increase in an enzyme-altered foci (21). Such foci had

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[4] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DEN, diethylnitrosamine; i.g., intragastric.

[5] P. A. Holmes, J. H. Rust, W. R. Richter, and A. M. Shefner. Long-term effects of TCDD and HCDD in mice and rats. Presented at the International Conference on Health Effects of Halogenated Aromatic Hydrocarbons, New York City, New York Academy of Sciences, June 24 to 27, 1978.

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Table 1

Promoting effect of TCDD on hepatocarcinogenesis by a single dose of DEN and PH[a]

Female rats (200 g) were intubated with DEN where shown. Seven days later, TCDD (injected s.c.) or phenobarbital (0.05% in the diet) administration was begun and was continued for 28 weeks, at which time the animals were sacrificed, and the livers were examined. The low and high doses of TCDD were 0.14 and 1.4 μg/kg/2 weeks, respectively, administered s.c. DEN was given at a dose of 10 mg/kg. See text for further details.

Group	Treatment	No. of animals	No. of enzyme-altered foci/cu cm of liver	Mean vol of enzyme-altered foci (cu mm)	% liver vol occupied by foci	N. of rats with carcinoma
1	PH + DEN	4	309 ± 98[b]	0.02	0.7	0
2	PH + TCDD (low dose)	4	34 ± 17	0.05	0.2	0
3	PH + TCDD (high dose)	5	25 ± 7	0.04	0.1	0
4	PH + phenobarbital	4	56 ± 13	0.01	0.1	0
5	PH + DEN + TCDD (low dose)	5	1068 ± 166	0.08	9.0	0[c]
6	PH + DEN + TCDD (high dose)	7	871 ± 66	0.49	43.0	5[d]
7	PH + DEN + phenobarbital	10	533 ± 103	0.15	6.0	8

[a] PH, partial hepatectomy.
[b] Mean ± S.D.
[c] Three rats exhibited neoplastic nodules in the liver.
[d] One rat exhibited neoplastic nodules in the liver.

been reported earlier to result from DEN administration (8) and are thought to be precursors of hepatocellular carcinomas (28, 31). Animals subjected only to partial hepatectomy and DEN develop far fewer enzyme-altered foci and no liver carcinomas.

For the quantitative analysis of the results per unit volume, we have used relationships established in the field of stereology (7) including quantitative stereology (38), quantitative metallography (36), and quantitative microscopy (5). The application of these established mathematical techniques to the computation of the number, volume, and size distribution of enzyme-altered foci per unit volume from measurements made on 2-dimensional histological sections was recognized and adapted for this purpose by H. A. Campbell. The method of Johnson (12) as extended and modified by Saltykov (30, 37) was very useful in calculating the foci size distribution of the foci permitting the estimates of foci number made from the method of Scherer *et al.* (32) to be placed on a firm quantitative basis.

In this report, we examine the promoting effect of TCDD on this 2-stage model of liver cancer and compare it with the effect of phenobarbital.

MATERIALS AND METHODS

Female Charles-River rats weighing 200 to 250 g were subjected to a 70% hepatectomy according to the procedure of Higgins and Anderson (11). A single i.g. intubation of DEN (10 mg/kg; Eastman Organic Chemicals, Rochester, N. Y.) in water was administered 24 hr later according to the protocol of Scherer and Emmelot (31). The animals were divided into treatment groups (Table 1); all of the groups were subjected to partial hepatectomy, but only 4 groups received DEN. The rats in Group 1 were given DEN and then maintained on standard laboratory chow for the remainder of the experiment (32 weeks). The rats in groups 2 and 3 received no DEN, but starting 1 week after hepatectomy they received biweekly s.c. injections of TCDD (0.14 or 1.4 μg/kg, respectively) in corn oil for a period of 28 weeks. (The TCDD was provided by Dow Chemical Co., Midland, Mich., as Lot 851-144-2, and it was 98.6% pure as determined by gas-liquid chromatography.) Groups 5 and 6 received DEN, and 1 week later they were initiated on a regimen of 14 biweekly injections of TCDD (0.14 or 1.4 μg/kg, respectively). The animals in Group 4 received 0.05% sodium phenobarbital in the diet starting 1 week after partial hepatectomy for 28 weeks, and the animals in Group 7 received DEN and then one week later also were given 0.05% sodium phenobarbital in the diet for the duration of the experiment. At the end of the experiment, the rats were killed, and sections of the liver were removed and frozen on solid CO_2. Serial sections of the frozen blocks of liver were cut and stained consecutively for glucose-6-phosphatase, canalicular ATPase, and γ-glutamyl transpeptidase (22) and with hematoxylin and eosin. The area of each liver section was measured using a planimeter. Photographs were taken of each histochemically stained section, and the number of enzyme-altered foci were determined from tracings of the photographic enlargements made on transparent plastic. By appropriate overlaying of the 3 transparencies, one for each enzyme stain, the total number of enzyme-altered foci per cu cm of liver could be calculated by use of the formula:

$$N_v = \frac{1}{r_1} + \frac{1}{r_2} + \frac{1}{r_3} + \cdots \frac{1}{r_n} \Big/ \pi A$$

where N_v is the number of foci per cu cm of liver; r_1, r_2, r_3, and r_n are the radii in cm of the foci transections; and A is the area in sq cm of the liver sections evaluated. This relationship has been reported by Drinkwater *et al.* (6)[6] and by Fullman (9). The percentage of the enzyme-altered cells in the entire liver population was calculated from the fact that the ratio of the area of the foci sections to the area of the liver tissue sections is identical to the ratio of the volume of the foci to the volume of the liver (43). Hepatocarcinomas were diagnosed by standard histopathological criteria.

RESULTS

As seen in Table 1, animals that were partially hepatectomized and then given only TCDD (Groups 2 and 3) or phenobarbital (Group 4) developed relatively few enzyme-altered foci. Hepatectomized animals receiving only DEN developed a substantial number of foci (Group 1), but the number of such

[6] N. R. Drinkwater, M. R. Moore, E. C. Miller, and J. A. Miller. Methods for the quantitative estimation of histochemically detectable foci of altered liver cells in carcinogen-treated animals, submitted for publication.

foci increased approximately 3-fold when these animals then received TCDD (Groups 5 and 6) or phenobarbital (Group 7) for the next 7 months. Most significant was the production of well-differentiated hepatocellular carcinomas in the high-dose TCDD-DEN-treated animals (Groups 5 and 6), whereas no neoplasms were observed in animals that received only DEN (Group 1) or only TCDD (Groups 2 and 3). Rats subjected to partial hepatectomy alone showed no foci (data not shown).

As can be seen from Table 1, the number of enzyme-altered foci per cu cm liver in animals of Group 5 was significantly higher than that for those in Group 6. We do not feel that this indicates that the lower dose of TCDD (equivalent to 0.01 μg/kg/day) is more effective in promoting liver tumors than the higher dose (0.1 μg/kg/day) but rather that the larger foci which occurred after 7 months on the higher dose of TCDD were the result of fusion of several of the foci, thus accounting for a lower total number in livers of animals on the higher dose for the same period of time. A comparable but somewhat lower number of foci than induced with the higher dose of TCDD was produced by the feeding of 0.05% phenobarbital following DEN-partial hepatectomy at the dose used in these experiments (Group 7). Thus, TCDD administration is similar to the promoting effect of phenobarbital in increasing the number of enzyme-altered foci but does not show any effect of dose on the number of foci produced in the ranges used in this study. Almost twice the number of enzyme-altered foci were produced by TCDD (0.01 μg/kg/day) as were produced by phenobarbital (0.05% in diet), but the total molar dose of the former was approximately 1 million times less.

While both dose levels of TCDD resulted in the same number of enzyme-altered foci, the higher dose level caused a marked increase in the total number of cells or volume occupied by the enzyme-altered foci in the livers of animals in Groups 5 and 6 (Table 1). This result is consistent with the concept that the promoting action of TCDD enhances the growth of the cells in the foci, which is reflected by a greater proportion of foci exhibiting phenotypes of ATPase and glucose-6-phosphatase deficiency combined with the presence of γ-glutamyl transpeptidase. Pugh and Goldfarb (27) have earlier shown that such a phenotype is characteristic of foci exhibiting the largest number of cells in DNA synthesis.

It was evident from observation of the transparent overlays for the 3 enzymes that many of the foci as well as the larger carcinomas exhibited heterogenous phenotypes of altered enzyme activities. The use of serial sections and composites of the transparent overlays for the enzymes allowed us to quantitate the number of enzyme-altered foci of each of the 7 possible phenotypes. Table 2 presents these results. The partially hepatectomized animals receiving DEN only (Group 1) or the TCDD only (Groups 2 and 3) exhibited a greater percentage of their foci as a single enzyme alteration when compared to the PH plus DEN plus TCDD animals (Groups 5 and 6). The percentage of foci exhibiting alterations in all 3 enzymes was 3- to 7-fold higher in the DEN-TCDD-treated animals than in the other groups.

DISCUSSION

In the present study, we have found that enzyme-altered foci induced in rat liver by partial hepatectomy and DEN were greatly increased in number, total volume, and phenotypic

Table 2

Number and percentage of distribution of phenotypes of enzyme-altered foci

See legend of Table 1 and text for further details.

Group	Treatment	GT[a]	AP	GP	GT, AP	GT, GP	AP, GP	GT, AP, GP	Totals
1	PH + DEN	118 [62, 244, 87, 77][b] (38)[c]	89 [30, 146, 78, 101] (29)	82 [8, 119, 26, 176] (27)	6 [0, 8, 5, 10] (2)	0 [0, 0, 0, 0] (0)	1 [0, 0, 5, 0] (1)	14 [0, 32, 13, 10] (4)	309 [100, 548, 213, 373]
2	PH + TCDD (low dose)	17 [0, 33, 35, 0] (50)	4 [10, 5, 0, 0] (12)	4 [0, 14, 0, 0] (12)	0 [0, 0, 0, 0] (0)	2 [7, 0, 0, 0] (6)	6 [0, 25, 0, 0] (18)	1 [0, 0, 5, 0] (3)	34 [18, 81, 35, 0]
3	PH + TCDD (high dose)	11 [5, 0, 0, 10, 41] (44)	4 [0, 13, 7, 0, 0] (16)	4 [0, 0, 0, 10, 8] (16)	0 [0, 0, 0, 0, 0] (0)	0 [0, 0, 0, 0, 0] (0)	5 [9, 0, 18, 0, 0] (20)	1 [0, 0, 3, 0, 0] (4)	25 [14, 13, 28, 20, 49]
4	PH + PB	20 [40, 38, 8, 15, 0] (36)	13 [18, 19, 6, 0, 24] (24)	19 [23, 16, 7, 41, 9] (34)	0 [0, 0, 0, 0, 0] (0)	0 [0, 0, 0, 0, 0] (0)	2 [3, 7, 0, 0, 0] (4)	1 [3, 3, 0, 0, 0] (2)	56 [86, 83, 20, 56, 33]
5	PH + DEN + TCDD (low dose)	416 [356, 641, 474, 442, 167] (39)	171 [207, 210, 131, 240, 65] (16)	224 [223, 203, 239, 298, 160] (21)	66 [86, 117, 46, 65, 17] (6)	46 [34, 114, 19, 43, 22] (4)	62 [69, 40, 103, 77, 23] (6)	83 [155, 137, 35, 73, 14] (8)	1068 [1129, 1462, 1046, 1237, 467]
6	PH + DEN + TCDD (high dose)	207 [270, 349, 178, 236, 58, 99, 257] (24)	86 [127, 42, 75, 66, 45, 90, 157] (10)	177 [127, 80, 163, 103, 323, 124, 316] (20)	54 [63, 106, 65, 39, 10, 47, 51] (6)	83 [46, 104, 111, 106, 23, 65, 126] (10)	70 [42, 15, 47, 42, 99, 146, 101] (8)	194 [121, 204, 159, 233, 105, 315, 220] (22)	871 [796, 900, 798, 873, 663, 887, 1227]
7	PH + DEN + PB	318 [382, 696, 664, 610, 416, 71, 96, 17, 71, 158] (60)	73 [63, 42, 132, 45, 95, 45, 16, 66, 161, 66] (14)	29 [62, 44, 33, 30, 19, 7, 13, 18, 17, 49] (5)	47 [72, 92, 92, 76, 48, 2, 16, 12, 30, 31] (9)	18 [29, 17, 37, 25, 25, 0, 13, 0, 11, 26] (3)	14 [9, 0, 4, 7, 47, 7, 9, 18, 15, 27] (3)	32 [32, 20, 24, 13, 71, 51, 15, 7, 47, 85] (6)	533 [649, 912, 986, 808, 721, 139, 179, 139, 352, 442]

[a] GT, γ-glutamyl transpeptidase positive; AP, canalicular ATPase negative; GP, glucose-6-phosphatase negative; PH, partial hepatectomy; PB, sodium phenobarbital.
[b] Numbers in brackets, individual values of each of the foci of that phenotype in the liver of each animal used.
[c] Numbers in parentheses, average percentage distribution of the phenotype with that treatment.

heterogeneity by the administration of TCDD. A significant incidence of hepatocellular carcinomas (5 of 7) was observed in the DEN-treated rats which were given the high dose of TCDD (1.4 μg/kg biweekly), but no carcinomas were seen in the rats treated only with DEN (0 of 4) in confirmation of previous results (21, 34). The rats and the TCDD dosage regimen used in this study were chosen to resemble closely the conditions in the 2-year feeding study by Kociba *et al.* (15). The rats in the present study, initiated with DEN and then given TCDD, developed a much higher incidence of liver cancer in a much shorter time period (28 weeks) than did those maintained on a diet of TCDD for 104 weeks in the study by Kociba *et al.*

In the absence of convincing evidence that TCDD is a mutagen or that it covalently binds to DNA to any appreciable extent and in light of the present results that TCDD enhances DEN-initiated hepatic carcinoma, it seems a reasonable hypothesis that all the tumors associated with the chronic administration of TCDD arise from its promoting activity of cells already "initiated" by exposure to the environment.

Boutwell (4) has suggested that promoting agents act to alter gene expression, and studies of one of the best known promoting agents in skin, tetradecanoyl phorbol ester, have repeatedly demonstrated its relative metabolic inertness and lack of covalent interaction with DNA. In conformity with this concept, TCDD has been shown to bind reversibly to a specific cytosol receptor, and the ligand-receptor complex initiates the coordinate expression of a number of genes (25).

The characteristic toxic responses of TCDD have recently been shown to be mediated through the cytosol receptor (24), and it is possible that its action as a promoter of hepatocellular carcinogenesis may also be mediated by its stereospecific binding to the receptor and the coordinated gene expression that ensues. The extreme effectiveness of this compound in its promoting action suggests that the relative strength of other promoting agents in the liver and probably other organs will vary by many orders of magnitude just as can be seen in the potency of chemical carcinogens (16). Furthermore, such an effective promoting agent might well be expected to be able to promote cells initiated by ambient environmental conditions such as diet, background radiation, or other factors (Table 1, Groups 2, 3, and 4), giving its effects the semblance of a complete carcinogen.

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