Liver Tumor-promoting Activity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) in TCDD-sensitive and TCDD-resistant Rat Strains


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ABSTRACT

Risk assessment of dioxins is currently based on induction of liver tumors in rats. The toxicity of dioxins is characterized by large sensitivity differences among animal species and even strains of the same species, which complicates the risk assessment. The significance of these differences in dioxin-induced carcinogenicity is not known. We therefore studied the liver tumor-promoting activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the sensitive Long-Evans (L-E) and the resistant Han/Wistar (H/W) rats differing >1000-fold in their sensitivity to the acute lethality of TCDD. Female rats were partially hepatectomized, initiated with nitrosodithioline, and treated with TCDD for 20 weeks. Altered hepatic foci (AHF) were stereologically quantitated using glutathione S-transferase P as a marker. AHF were significantly (P < 0.001) and dose dependently increased in L-E rats at 10 and 100 ng/kg/day, but in H/W rats only at 1000 ng/kg/day and above, indicating a remarkable (~ 100-fold) sensitivity difference between L-E and H/W rats. The same sensitivity difference but 10-fold less foci were observed between nonhepatectomized/omitted L-E and H/W rats. Induction of AHF was related to hepatotoxicity but not to cytochrome P450A1 activity in the liver. Liver TCDD concentrations were similar in both strains. H/W rats are exceptionally resistant to induction of AHF by TCDD, and the resistance is associated with an altered transactivation domain of the aryl hydrocarbon receptor. Genetic differences may account for significant interindividual/intraspecies sensitivity differences in dioxin-induced carcinogenesis. Understanding the role of transactivation domain of the aryl hydrocarbon receptor in carcinogenesis is therefore likely to improve dioxin risk assessment.

INTRODUCTION

TCDD3 is one of the most toxic environmental pollutants and the model compound for CDDs. Because of their lipophility, stability, and resistance to biodegradation, dioxins bioaccumulate in the food chain and are ubiquitously present in human adipose tissue as well as in mother’s milk. Several studies have revealed that these compounds share common mechanisms of action. They elicit a variety of common biochemical and toxic responses, including specific binding to the cytosolic AHR (reviewed in Refs. 1 and 2), and the only major difference among CDD congeners seems to be in their relative potencies.

TCDD induces a variety of biological responses ranging from induction of cytochrome P-450 1A (CYP1A) to reproductive and developmental defects, immunotoxicity, thymus atrophy, epithelial disorders, liver damage, wasting syndrome, and cancer (reviewed in Ref. 3). In a recent re-evaluation of the carcinogenicity of TCDD, the IARC has upgraded the classification from possible human carcinogen (group 2B; Ref. 4) to human carcinogen (group 1; Ref. 5). The evidence for carcinogenicity of TCDD was considered limited in humans and sufficient in experimental animals. In lifetime bioassays, TCDD was found to be a multisite carcinogen in both genders of all animal species studied (rats, mice, and hamsters), causing several tumor types at sites distant from the point of administration. On the other hand, TCDD does not interact with DNA, and despite a few conflicting reports, it is not considered directly genotoxic (reviewed in Ref. 5). Accordingly, in two-stage initiation-promotion models, TCDD seems to lack tumor-initiating activity. There is, however, an overflow of data indicating that TCDD is a potent tumor promoter in rat and mouse liver and lung, as well as in mouse skin (reviewed in Ref. 5; see examples in Refs. 6–11). TCDD has also proved to be positive in cell transformation assays measuring tumor-promoting activity in cultured rodent or human cells in vitro (12, 13).

The current risk assessment of dioxins has mainly been based on a 2-year carcinogenicity bioassay with TCDD in Sprague Dawley rats (14). It uses the incidences of liver neoplasms in females as the critical end point of toxicity. Nevertheless, the mechanisms of TCDD-induced carcinogenesis are incompletely understood. In the absence of direct genotoxicity, several possible mechanisms have been proposed. Specific binding of TCDD to AHR and the subsequent induction of gene expression seems to have an important role in mediating a variety of toxic effects of TCDD (15–18). Activation of AHR may also be involved in the carcinogenicity of TCDD, but the details of its role in different stages of carcinogenesis remain unclear (5). In rats (but not in other rodent species), females seem to be more sensitive to the hepatocarcinogenicity of TCDD. Ovarian hormones are likely to be involved, because ovariec-tomy has been shown to inhibit promotion of TCDD-induced preneoplastic foci and liver tumors (8). Indirect genotoxicity attributable to TCDD-induced CYP1A2- or CYP1B1-mediated metabolism of estradiol to catechol estrogens and subsequently increased formation of reactive oxygen species has been suggested (19, 20). Possible epigenetic mechanisms of TCDD-induced hepatocarcinogenesis include selectively increased cell proliferation and, more importantly, reduced apoptosis in focal cell populations, resulting in net growth of AHF (21), down-regulation of

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3 The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; CDD, chlorinated dibenzo-p-dioxin; AHR, aryl hydrocarbon receptor; MNPCE, micronucleated bone marrow polychromatic erythrocyte; MNRET, micronucleated peripheral blood reticulocyte; GC-MS, gas chromatography-mass spectrometry; LOAEL, lowest observable adverse effect level; NOEL, no-effect level.
epidermal growth factor receptor in liver (22), altered gap-junctional communication (23, 24), and increased expression of the proto-oncogene product ras p21 protein (25). Furthermore, there appears to be a distinct correlation between hepatotoxicity and the development of hepatocellular neoplasms (14, 26).

A characteristic feature in the toxicity of dioxins is exceptionally large sensitivity differences among animal species or even strains of the same species. These differences, although best illustrated for acute lethality, together with lack of understanding of the critical mechanisms, highly complicate the risk assessment of dioxins. An animal model based on >1000-fold sensitivity difference in acute lethality of TCDD between two rat strains has been established in our laboratory. L-E (Turku/AB) rats are highly sensitive, having an LD₅₀ of 10 μg/kg. H/W (Kuopio) rats, on the other hand, are the most TCDD-resistant mammals known with an LD₅₀ of >9600 μg/kg (27, 28). The toxicokinetics of TCDD is nearly similar in these strains (29). No substantial differences between these rat strains could be detected in the amount of hepatic AHR, binding affinity of TCDD to AHR, or specific binding of the activated AHR to DNA (17, 30). However, the AHR of H/W rats was shown recently to harbor a point mutation, which results in an insertion/deletion type alteration at the 3′ end of the coding region of cDNA (16). At the protein level, the molecular mass of the receptor is smaller because of a loss of amino acids from the transactivation domain. Although this deviant AHR seems to account for the tremendous resistance of H/W rats to acute lethality and intermediate resistance to liver toxicity (18), H/W rats and L-E rats show nearly similar sensitivity to induction of CYP1A1 activity, thymic atrophy, embryotoxicity, or decreases in serum thyroxine and melatonin levels (reviewed in Ref. 3). This animal model is therefore highly useful for studying the significance of intraspecies sensitivity differences in end points critical for risk assessment of dioxins. The objective of the present study was to compare the sensitivity of L-E and H/W rats to the liver tumor-promoting activity of TCDD in relation to liver TCDD concentrations and other end points of toxicity.

**MATERIALS AND METHODS**

**Test Chemicals.** TCDD (CAS 1746-01-6; molecular weight, 321.9; purity, >99% as analyzed by GC-MS) was purchased from UFA Oil Institute (Ufa, Russia). It was dissolved in corn oil (Sigma Chemical Co., St. Louis, MO). NDEA was obtained from Sigma.

**Animals.** Inbred female L-E (Turku/AB) and outbred female H/W (Kuopio) rats were obtained from the breeding colony of the National Public Health Institute (Kuopio, Finland), kept in an specific pathogen-free barrier unit. The animals are regularly subjected to health surveys consisting of serological and bacteriological screening as suggested by the Federation of European Laboratory Animal Science Associations (FELASA) (31). These surveys indicated that the animals were free of typical rodent pathogens. The rats were 5 weeks of age and weighed 70.1 ± 7.8 g (L-E; mean ± SD) or 81.7 ± 3.9 g (H/W) at hepatectomy. They were housed in stainless steel, wire-bottomed cages 5 rats/cage and given standard pelleted R36 feed (Ewos, Söderås, Sweden), and tap water ad libitum. The room was artificially illuminated from 7 a.m. to 7 p.m., the ambient temperature was 21.5 ± 1°C, and relative humidity was 55 ± 10%.

**Experimental Design.** Experimental groups and dosages are shown in Table 1. In addition to the main study, a separate experiment (satellite groups) of high-dose levels was carried out on the resistant H/W rats. In this experiment, H/W rats were exposed to total doses of 170 μg/kg (the same as the highest dose of the main study) or 1700 μg/kg (given either as weekly doses or as a single dose).

A part of the rats were assigned to the complete initiation-promotion treatment including a PH and an initiation with NDEA (+/+), followed by promotion with TCDD, whereas the rest of the animals were only treated with TCDD (−/−). The animals assigned for PH and initiation were two-thirds partially hepatectomized (32) under diethylether (Riedel-de-Haen, Seelze, the Netherlands) anesthesia, and 24 h later, the animals were initiated with a single dose of 30 mg/kg NDEA i.p. Starting 5 weeks after PH, the rats were administered TCDD by s.c. injections (2 ml/kg) once a week for 20 weeks. To rapidly achieve the kinetic steady state, the first dose was a loading dose, which was five times as high as the 19 consecutive maintenance doses (7). Control groups were administered corn oil. In addition, untreated control groups of both strains (both with and without PH + NDEA) were included in the study. The rats were observed daily, and they were weighed weekly.

At termination, the rats were anesthetized with CO₂/O₂ (70/30%). Blood samples were drawn from the left ventricle, and the rats were exsanguinated by cutting the aorta. The rats were subjected to cross necropsy tissue sampling for preneoplastic hepatic foci and histopathology. The weights of liver and thymus were recorded.

The study protocol was approved by the Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government, and it was in accord with institution guidelines.

**Analysis of AHF.** Liver samples were fixed in ice-cold acetone, embedded in paraffin, cut to the thickness of 4 μm, and immunohistochemically stained for GST-P, the placental isoform of GST. GST-P-positive foci were quantified using a Leica Aristoplan microscope connected to a Quantimet 570

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**Table 1. Treatment groups, doses of TCDD, and number of animals**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Maintenance dose</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total dose (μg/kg)</td>
<td>Loading dose (μg/kg)</td>
<td>Weekly dose (μg/kg)</td>
</tr>
<tr>
<td>Main study</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Untreated controls</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1. Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. TCDD</td>
<td>0.17</td>
<td>0.035</td>
<td>0.007</td>
</tr>
<tr>
<td>3. TCDD</td>
<td>1.7</td>
<td>0.35</td>
<td>0.07</td>
</tr>
<tr>
<td>4. TCDD</td>
<td>17</td>
<td>3.5</td>
<td>0.7</td>
</tr>
<tr>
<td>5. TCDD</td>
<td>170</td>
<td>35</td>
<td>7</td>
</tr>
<tr>
<td>Satellites</td>
<td>170</td>
<td>35</td>
<td>7</td>
</tr>
<tr>
<td>SS. TCDD</td>
<td>170</td>
<td>35</td>
<td>7</td>
</tr>
<tr>
<td>S6. TCDD</td>
<td>170</td>
<td>350</td>
<td>7</td>
</tr>
</tbody>
</table>

* PH + initiation with NDEA.

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Biological Assays. Plasma samples for activities of ALAT, ASAT, and GGT were analyzed according to the guidelines of the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1974 and 1979) using a selective chemistry analyzer (Kone Specific; Kone Instruments, Espoo, Finland).

Hepatic CYP1A1 activity was measured as O-dealkylation of 7-ethoxyresorufin (EROD) in S9 fraction using a Shimadzu RF-5000 spectrofluorometer (33, 35).

Histopathology. Liver samples were preserved in 10% neutral buffered formalin, dehydrated, embedded in paraffin wax, and cut to a thickness of 5 μm. The tissue slices were mounted on glass slides, stained with Mayer’s H&E, and examined using light microscope by one pathologist (V-M. K.). An extensive response was used where appropriate.

Analysis of Micronucleated Erythrocytes. In the main study, MNPCe and MNRET frequencies were studied in 5/−− and 5/0/+/+ rats of both strains. In addition, micronuclei were analyzed in PCEs and in RETs of 1/+/+ rats of the satellite groups. For the analysis of MNPCe, micrometers were dissected out at necropsy and processed according to Schmid (36). For the analysis of MNRETs, blood samples were collected by cardiac puncture in anesthetized animals at necropsy. The smear slides were fixed in methanol, air dried, and stained with acridine orange as described by Hayashi et al. (37). One thousand bone marrow PCEs and one thousand RETs per animal were analyzed for micronuclei by fluorescent microscopy. The proportion of PCEs to normochromat cells in the total erythrocyte population of bone marrow was scored by counting the cells until the score for one cell type reached one thousand. The frequency of immature erythrocytes in peripheral blood (RETs) was analyzed in 500 (main study) or in 200 (satellite groups) erythrocytes per rat. The analyses were done on coded slides by one microscopist (J. M-P.).

Analysis of Liver TCDD Concentrations. Liver TCDD concentrations for the main study were determined in five rats/treatment group at the National Public Health Institute (Kuopio, Finland). The weighted samples were spiked with 840 pg of 13C-labeled TCDD/E standard (Cambridge Isotope Laboratories, Inc., Woburn, MA), mixed with anhydrous Na2SO4, dried in an oven at 80°C, put at the top of the acidic silica gel column, and cleaned and analyzed according to Kiviranta et al. (39).

Statistics. For comparisons between groups of the main study the data of continuous variables with presumed normal distribution were tested for homogeneity of group variances using the Bartlett’s test. If the variances were homogeneous such as or after appropriate transformation (at the level of 0.01), comparisons between treatment groups were performed using the one-way ANOVA, followed by the Least Significant Difference test. If the variances were heterogeneous, comparisons were made using the nonparametric Kruskal-Wallis ANOVA, followed by the Mann-Whitney U test. Incidences of histopathological findings were analyzed using Fisher’s exact test. Frequencies of micronucleated cells and immature erythrocytes were analyzed by the Mann-Whitney U test. All tests were two-sided.

RESULTS

Body Weight Development and Mortality. Body weight gain was dose dependently decreased in TCDD-treated L-E rats at 1.7 μg/kg and above and in TCDD-treated H/W rats at 17 μg/kg and above (Table 2). +/+ rats gained more weight than −− rats, and this difference was more conspicuous in L-E rats. In +/− L-E rats, the increase in body weight during the TCDD treatment period (from week 5 to week 25 of the study) was 78.3% (P < 0.001) and 44.4% (P < 0.001) of controls at 1.7 and 17 μg/kg, respectively, whereas in the −− L-E rats, the difference was significant only at 17 μg/kg (63.3% of controls; P < 0.001). In H/W rats, the increase in body weight at 17 and 170 μg/kg was 81.4 (P < 0.05) and 56.3% (P < 0.001) of controls for +/+ animals and 82.6 and 49.9% (P < 0.001) of controls for −− animals, respectively. Body weight development of the H/W satellite groups receiving 170 or 1700 μg/kg TCDD was added as recovery standard. Separation of the CDDs and quantification were carried out by capillary GC-MS with a high resolution mass spectrometer (Finnigan MAT 95 s, Argentueil, France) in EI-/SIM-mode at a resolution of r = 10,000. Operating conditions for GC (HP 6890 Series) were according to Schramm et al. (38). The quality of all solvents was for residue analysis (Promochem, Wesel, Germany), and the other chemicals were of the highest purity available.

Liver TCDD concentrations for the satellite groups were determined for two to eight rats/treatment group at the National Public Health Institute (Kuopio, Finland). The weighted samples were spiked with 840 pg of 13C-labeled TCDD/E standard (Cambridge Isotope Laboratories, Inc., Woburn, MA), mixed with anhydrous Na2SO4, dried in an oven at 80°C, put at the top of the acidic silica gel column, and cleaned and analyzed according to Kiviranta et al. (39).

Table 2. Body weight gain and mortality during TCDD treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Total dose (μg/kg)</th>
<th>PH/NDEA</th>
<th>Body weight gain (g)a</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H/W</td>
<td>L-E</td>
</tr>
<tr>
<td>Main study</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Control</td>
<td>0</td>
<td>++/+</td>
<td>89.9 ± 4.7</td>
<td>79.4 ± 2.91</td>
</tr>
<tr>
<td>2. TCDD</td>
<td>0.17</td>
<td>+/+</td>
<td>81.6 ± 4.82</td>
<td>59.4 ± 2.46</td>
</tr>
<tr>
<td>3. TCDD</td>
<td>1.7</td>
<td>+/+</td>
<td>90.0 ± 8.32</td>
<td>54.6 ± 2.87</td>
</tr>
<tr>
<td>4. TCDD</td>
<td>17</td>
<td>+/+</td>
<td>73.2 ± 3.0</td>
<td>35.2 ± 4.50</td>
</tr>
<tr>
<td>5. TCDD</td>
<td>170</td>
<td>+/+</td>
<td>67.2 ± 2.65</td>
<td>34.2 ± 3.25</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>−/−</td>
<td>+/+</td>
<td>84.5 ± 14.9</td>
<td>81.4 ± 3.68</td>
</tr>
<tr>
<td>Satellites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3. TCDD</td>
<td>170</td>
<td>+/+</td>
<td>28 ± 10.2</td>
<td>10</td>
</tr>
<tr>
<td>S6. TCDD</td>
<td>170</td>
<td>+/+</td>
<td>25.8 ± 9.0</td>
<td>52.9</td>
</tr>
<tr>
<td>1700 (single dose)</td>
<td>−/−</td>
<td>+/+</td>
<td>15.8 ± 10.1</td>
<td>60</td>
</tr>
</tbody>
</table>

a PH + initiation with NDEA.

b Mean ± SE.

c P < 0.001 versus controls.

d P < 0.05 versus controls.
TCDD was also markedly below controls. The magnitude of the effect observed in L-E rats at 1.7 and 17 μg/kg was comparable with that in H/W rats at 17 and 170 μg/kg, respectively. This indicates about a 10-fold sensitivity difference in body weight development between the strains.

In the main study, one /+/ L-E rat of 10 from the 17 μg/kg TCDD group was killed in moribund condition on week 22 of the study (Table 2). This rat showed a characteristic manifestation of TCDD intoxication with signs of wasting syndrome (a body weight loss by 26%, loss of body fat) and gastrointestinal hemorrhagia. In the satellite groups, there were 16 deaths, all of which were also considered treatment related. These results suggest that H/W rats are less resistant to the long-term than to acute (42-day) mortality of TCDD (27, 28).

Quantitative Stereology of GST-P-positive Hepatic Foci. Results from the stereological evaluation of altered foci are shown in Fig. 1. No foci were detected in untreated /+/ control rats of either strain, Fig. 1. Dose responses of the presence of GST-P positive foci in liver expressed as volume fraction of liver occupied by the foci (A and B) and the number of foci per cm³ (C and D) in nonhepatectomized/ noninitiated (−/−, left panel, open symbols) and partially hepatectomized/initiated (+/++, right panel, solid symbols) L-E and H/W rats. Note the different scales in left and right panels. Group means are shown; bars, SE (for n, see Table 1). UC, untreated controls. * P < 0.05; ** P < 0.01; *** P < 0.001 versus controls.

Table 3 Incidence of liver histopathology findings in the main study

<table>
<thead>
<tr>
<th>Treatment group (total dose μg/kg)</th>
<th>H/W</th>
<th>L-E</th>
<th>PH + initiation</th>
<th>L-E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.17</td>
<td>1.7</td>
<td>17</td>
</tr>
<tr>
<td>No hepatectomy, no initiation</td>
<td>UC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of animals examined</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Foci +**</td>
<td>4 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foci +++</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Foci All</td>
<td>4 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory foci</td>
<td>1</td>
<td>4 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrotic foci +</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Necrotic foci ++</td>
<td>2</td>
<td>3</td>
<td>1</td>
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<td>Fibrosis +</td>
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<td></td>
<td></td>
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<tr>
<td>Fibrosis ++</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>Multinucleated hepatocytes +</td>
<td>3</td>
<td>2</td>
<td>4 2</td>
<td>1</td>
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<tr>
<td>Multinucleated hepatocytes All</td>
<td>1</td>
<td>5 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic vacuolization</td>
<td>1</td>
<td>2</td>
<td>1</td>
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<tr>
<td>Cystic degeneration</td>
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<tr>
<td>Mitotic figures +</td>
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<td></td>
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<tr>
<td>Hepatocellular adenoma</td>
<td>1</td>
<td>5 2</td>
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<td>Bile duct dilatation</td>
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<td>Bile duct hyperplasia +</td>
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<tr>
<td>Bile duct hyperplasia ++</td>
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<td></td>
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<tr>
<td>Bile duct hyperplasia All</td>
<td>3</td>
<td>1</td>
<td>6 1</td>
<td>1</td>
</tr>
<tr>
<td>Extramedullary hematopoiesis</td>
<td>5</td>
<td>1</td>
<td>5 4</td>
<td>1</td>
</tr>
</tbody>
</table>

* Untreated controls.

Total dose (μg/kg).

+ , a mild response; ++, a moderate response; ++++, an extensive response.

Statistics: * P < 0.05; ** P < 0.01; *** P < 0.001 versus controls (Fisher’s exact test).

Multinucleated.
and the values were very low for −/+ vehicle control rats (mean volume fraction, 0.006 and 0.01%, and mean number of foci/cm², 26 and 46 in H/W and L-E rats, respectively). The volume fraction of foci was significantly (P < 0.001; t test) higher in both untreated and vehicle-treated +/+ control L-E rats than in similarly treated H/W rats (3.58 and 2.14% in untreated and vehicle-treated L-E rats, respectively, and 0.55 and 0.42% in untreated and vehicle-treated H/W rats, respectively). However, +/+ controls did not show any strain differences in the mean number of foci. Thus, PH/NDEA without TCDD resulted in a significantly larger volume of GST-P-positive foci in L-E rats than in H/W rats.

TCDD treatment resulted in a dose dependently increased volume fraction and number of GST-P-positive foci in both −/− and +/+ rats of both strains (Fig. 1). Comparison of the dose-response curves reveals that L-E rats were ~100-fold more sensitive to the promotion of foci by TCDD than H/W rats. In L-E rats, the volume fraction of foci and the number of foci/cm² were significantly (P < 0.001) dose dependently increased at 1.7 and 17 μg/kg, but in H/W rats only at 170 μg/kg (P < 0.01 for −/− rats and P < 0.001 for +/+ rats). The highest volume fraction of foci was observed in +/+ H/W satellites at 1700 μg/kg (18.5% of the liver volume occupied by foci; Fig. 1B). The number of foci, however, was rather low in this group (Fig. 1D). Also, the values for −/− H/W satellites were low (Fig. 1, A and C). In general, the focus parameters were about 10–20 times higher in +/+ than in −/− rats, but otherwise PH/NDEA had no influence on the dose-response characteristics or sensitivity to TCDD.

The higher background incidence of foci in +/+ L-E rats did not affect their sensitivity to TCDD-induced tumor promotion, because −/− rats responded similarly at the same dose levels. Accordingly, the sensitivity difference between L-E and H/W rats was the same (~100-fold) with and without PH/NDEA.

Liver Histopathology. A summary of histopathological findings is shown in Tables 3 and 4. Altered hepatic foci were observed in −/− L-E rats only at 1.7 and 17 μg/kg and in −/− H/W rats only at 170 and 1700 μg/kg. However, in +/+ rats, altered foci were present also in control and low-dosage groups. In +/+ H/W rats, there was a dose-related increase in the incidence of foci, and the severity of response (in terms of the abundance of foci per liver) was increased at 170 μg/kg and above. In +/+ L-E rats, on the other hand, all examined rats had foci, but the severity of the response was increased at 1.7 and 17 μg/kg. Hepatocellular adenoma/foci was observed in one (of 9 examined) +/+ L-E rats at 17 μg/kg and in 4 (of 9) and 3 (of 8) +/+ H/W satellite rats at 170 and 1700 μg/kg, respectively. A cholangiocarcinoma was found in one −/− H/W rat (of 8) that had received a single dose of TCDD at 1700 μg/kg. No other hepatic malignancies were observed.

Changes characteristic of TCDD-induced liver toxicity were detected mainly at 17 μg/kg in L-E rats and at 17 μg/kg and above in H/W rats. These changes included inflammatory and/or necrotic foci, fibrosis, multinucleated hepatocytes, cytoplasmic vacuolization, bile duct dilation, bile duct hyperplasia, and extramedullary hematopoiesis.

Plasma Enzyme Activities. Increased activities of ALAT, ASAT, and GGT in plasma are indicators of liver damage. Activities of these enzymes in plasma are shown in Fig. 2. In general, L-E rats were more sensitive than H/W rats exhibiting dose dependently increased enzyme activities in plasma at 1.7 (in most cases) and 17 μg/kg, whereas H/W rats typically responded only at 170 μg/kg and higher dose levels (satellites). Plasma ALAT activities were elevated only at 17 μg/kg in L-E rats (P < 0.001). In satellites, the highest elevation was observed at the 1700 μg/kg single-dose group. ASAT activities were significantly elevated at 1.7 and 17 μg/kg in L-E rats and at 170 μg/kg in H/W rats. The elevations of both ALAT and ASAT activities were more pronounced in −/− than in +/+ groups. In contrast, plasma GGT activities showed elevations only in +/+ groups. The elevations were significant (P < 0.001) at 1.7 and 17 μg/kg in L-E rats and at 170 μg/kg in H/W rats. In linear regression analysis, volume fractions of AHF were best (P < 0.001) correlated with plasma ASAT activities (r = 0.63, 0.71, 0.87 for +/+ L-E, −/− L-E, +/+ H/W, and −/− H/W rats, respectively), indicating an association between induction of foci and hepatotoxicity.

Liver EROD Activity. TCDD treatment resulted in a dose-dependant increase in liver EROD activity that was largely similar in both strains (P < 0.01 for all TCDD-treated groups; Fig. 3, A and B). The maximum EROD activity was measured at 1.7 μg/kg TCDD in L-E rats and at 17 μg/kg TCDD in H/W rats. At higher dose levels, the activity decreased, most likely because of overt toxicity and liver toxicity observed at 17 μg/kg in L-E rats and at 170 μg/kg and above in H/W rats.

Organ Weights. Body weight-related liver weights were dose-dependently increased in both strains (Fig. 3, C and D). The increases were significant at dose levels of 1.7 μg/kg and above in L-E rats and at 17 μg/kg and above in H/W rats. Thus, L-E rats were ~10-fold more sensitive, but this difference may reflect a more profound decrease in body weight gain in this strain. The maximum increase occurred at 170 μg/kg. At the highest dose levels, the increases were slightly more prominent in the PH/NDEA groups.

TCDD treatment resulted in dose dependently decreased body weight-related thymus weights in H/W and L-E rats (thymus weight in untreated L-E rats is only about one-third of that in untreated H/W rats; Fig. 3, E and F). Both strains showed similar sensitivity by
responding at the same dose levels. The decrease became statistically significant at 17 μg/kg, but it was more profound in L-E rats (19.8 and 24.4% of the control value in 2/2 and 1/1 L-E rats, respectively) than in H/W rats (63.6 and 72.6% of the control value in 2/2 and 1/1 H/W rats, respectively).

**Micronucleated Erythrocytes in Bone Marrow and Peripheral Blood.** Frequencies of MNPCEs in bone marrow and MNRETs in peripheral blood are shown in Fig. 4, A–D. The background amount of MNPCEs in bone marrow was 2–3-fold higher in L-E rats than in H/W rats. There was a tendency to a slight increase in MNPCEs at the highest doses, but the increase was significant only in 2/2 H/W rats at 170 μg/kg (P < 0.05). In peripheral blood, slight increases in MNRETs were observed in 1/1 H/W rats (including satellites) at 170 μg/kg and in 2/2 L-E rats at 17 μg/kg. The proportion of young erythrocytes (PCEs in bone marrow and RETs in peripheral blood) in the erythrocyte population is considered as an indicator of bone marrow toxicity, and decreased proportion of PCEs generally reflects suppression of erythropoiesis. In this study, however, TCDD treatment resulted in an increased proportion of PCEs and RETs, indicating stimulation of erythropoiesis (Fig. 4, E–H). In bone marrow, H/W rats showed more clear increases, especially in 2/2 groups (P < 0.01 at 170 μg/kg). The increases were more uniform in peripheral blood, achieving statistical significance at 17 μg/kg in L-E rats and at 170 μg/kg in H/W rats.

Increased frequency of MNPCEs generally indicate chromosomal damage. The increases, however, occurred at dose levels causing overt toxicity (reduced body weight development and hepatotoxicity) and stimulation of erythropoiesis. It is, therefore, possible that the observed induction of micronuclei is secondary to other toxic effects and does not represent a specific genotoxic effect of TCDD.

**Liver TCDD Concentrations.** Background liver concentrations of CDDs and polychlorinated dibenzofurans in untreated controls and vehicle controls at the end of the study were very low (range, 0.003–0.03 ng of WHO-TEq/g dry weight; WHO-TEq is TCDD equivalent quantity according to WHO), indicating lack of contamination in the animal room. Liver TCDD concentrations at the end of the study reflected accurately the doses administered (Fig. 5). There were no consistent differences between L-E and H/W rats or attributable to PH/NDEA. Analytical data make it possible to relate the tumorigenic effects with liver TCDD concentrations. For the induction of AHF, the LOAEL in L-E rats is 10 ng/kg/day (total dose, 1.7 μg/kg), corresponding to the liver TCDD concentration of 7.97 + 0.38 (mean + SE) ng/g dry weight (about 2.11 ng/g wet weight). The NOEL for the same response is 1 ng/kg/day (total dose, 0.17 μg/kg) and the corresponding liver TCDD concentration 0.61 + 0.04 ng/g dry weight (~0.16 ng/g wet weight). For H/W rats, the LOAEL is 1000 ng/kg/day (total dose, 170 μg/kg) and the liver TCDD concentration 626 + 41 ng/g dry weight (~166 ng/g wet weight), and NOEL 100 ng/kg/day (total dose 17 μg/kg) and liver TCDD concentration 78.1 + 4.7 ng/g dry weight (~20.7 ng/g wet weight).
DISCUSSION

The present study compared the liver tumor-promoting activity of TCDD in TCDD-sensitive L-E rats and the exceptionally resistant H/W rats, which have an altered transactivation domain of the AHR. AHF represent an early phase of carcinogenesis in the way to the development of malignant tumors, and data on preneoplastic hepatic lesions correlate well with the outcome of long-term carcinogenicity bioassays in rats (40 – 42). The study protocol using PH, initiation with a nonnecrogenic dose of NDEA (\(50 \, \text{mg/kg}\)), and the 20-week loading dose/maintenance dose promotion regimen has been developed and routinely used in our laboratory (7). GST-P is a superior phenotypic marker of TCDD-induced AHF (11, 42). The selected dose levels of TCDD covered the whole spectrum of biological effects ranging from slight induction of liver EROD activity to decreased body weight development and mortality in both strains. They also proved to cover the critical part of the dose responses for induction of AHF.

This study demonstrated a remarkable (\(100\)-fold) sensitivity difference between L-E and H/W rats in TCDD-induced development of AHF. This difference is essentially attributable to the exceptional resistance of H/W rats to the focus development, because the sensitivity of L-E rats did not differ from that reported earlier for female Sprague Dawley, Fischer 344, and Wistar rats (reviewed in Ref. 5). Furthermore, in agreement with the present L-E data, our previous studies with initiated Sprague Dawley rats using the same treatment protocol revealed increased induction of GGT- or GST-P-positive foci at the total dose levels of 1.1–24 \(\mu\text{g/kg}\) (maintenance dose, 6.3–143 ng/kg/day; Refs. 9, 24, 33, and 43). Thus, LOAEL for induction of AHF is in general \(\sim 10 \, \text{ng/kg/day}\) (or slightly below), and NOEL is \(\sim 1 \, \text{ng/kg/day}\). Moreover, dose responses for AHF in two-stage tumor promotion studies and development of hepatocellular adenomas in 2-year carcinogenicity bioassays are also very similar (44). It can be concluded that all other rat strains studied thus far, except H/W rats (LOAEL, 1000 ng/kg/day; NOEL, 100 ng/kg/day), are quite similar in sensitivity to the induction of AHF.

Toxicokinetic factors do not account for the resistance of H/W rats, because no strain differences in liver TCDD concentrations were detected. Liver TCDD concentrations in L-E and H/W rats were also similar to the steady-state concentrations reported earlier for Sprague Dawley rats at comparable dose levels (9, 10).

In previous studies, diverging sensitivity of C57BL/6 and DBA/2 mice to TCDD have been used in attempts to clarify the mechanism of TCDD-induced tumorigenicity. Lower binding affinity of the DBA/2 mouse AHR for TCDD is reflected in their \(10\)-fold higher resistance to a variety of characteristic TCDD-induced effects compared with the sensitive C57BL/6 mice. There is some indirect evidence that the AHR would have a role in promotion of skin tumors by TCDD, but the role of AHR in liver carcinogenesis has been less clear (5). An attempt to establish a rank order of sensitivity for liver tumor promotion among C57BL/6 and DBA/2 mice and their crosses (B6D2F\(_1\) mice) using a single dose level of TCDD (daily dose, 7.14 ng/kg/day) was not successful (45). Our results suggest an association...
between the exceptional resistance to TCDD-induced liver tumorigenesis and the altered transactivation domain of the AHR. This is in accord with our previous data, indicating that the resistance to TCDD-induced toxic effects (lethality, body weight loss, and hepatotoxicity) of H/W rats segregates with the altered Ahr allele (18).

Comparison of the outcome of carcinogenicity bioassays in rats and mice reveals no marked species differences in sensitivity to TCDD-induced liver carcinogenesis (reviewed in Ref. 5). On the other hand, hamsters that are very resistant to the lethality of TCDD did not develop liver tumors at total dose levels of up to 600 μg/kg (46). Squamous cell carcinomas of the facial skin, however, were observed at the highest dose level only. It is interesting to note that of all of the rodents studied, only H/W rats and hamsters share the relative resistance to the tumorigenic effect of TCDD. Furthermore, another common feature is the altered structure of the transactivation domain in both H/W rat and hamster AHR. In H/W rat AHR, there is a loss of

![Fig. 4. Dose responses of the frequency of micronucleated (MN) PCEs in bone marrow (A and B) and that of reticulocytes in peripheral blood (C and D), the proportion of polychromatic erythrocytes of all erythrocytes (polychromatic + normochromatic) in bone marrow (E and F), and the proportion of reticulocytes of all erythrocytes (reticulocytes + mature erythrocytes) in peripheral blood (G and H) of nonhepatectomized/noninitiated (−/−, left panel, open symbols) and partially hepatectomized/initiated (+/+ , right panel, solid symbols) L-E and H/W rats. Group means are shown; bars, SE (n = 5–10). +, P < 0.05; ++, P < 0.01 versus controls.]

![Fig. 5. Liver TCDD concentrations in individual nonhepatectomized/noninitiated (open symbols) and partially hepatectomized/initiated (solid symbols) L-E and H/W rats at the end of the study (n = 5 for the main study and 2–8 for the satellite groups).]
amino acids near the COOH-terminal end (16), whereas in the case of hamster AHR, the functionally essential Q-rich subdomain is substantially expanded (47).

In the present study, TCDD was found to dose dependently induce a small amount of GST-P-positive foci also in noninitiated L-E and H/W rats. It is noteworthy that the same (≈100-fold) sensitivity difference was detected also between noninitiated L-E and H/W rats, and the effect was observed at the same dose levels in initiated and noninitiated rats. Previous studies did not pay much attention to induction of foci by TCDD in noninitiated animals, apparently because of the lower response compared with that in initiated animals. There are only a few reports about increased generation of foci with some dose dependence (10) and time dependence (25, 48) in noninitiated rats. These foci were suggested to result from promotion of “spontaneously” initiated cells by TCDD. Furthermore, recent modeling of the experimental data have raised suggestions that in addition to its promoting activity, TCDD would have some initiating activity, possibly by an indirect mechanism (49, 50). Taken together, TCDD is able to induce a tumorigenic response in noninitiated animals with a potency similar to that observed with initiation.

According to the current view, TCDD is not a genotoxic compound (5). Nevertheless, increased frequencies of micronucleated erythrocytes observed at the highest dose levels of the present study indicate occurrence of some chromosomal damage. These dose levels caused overt toxicity and stimulation of erythropoiesis, as indicated by increased frequency of PCEs in bone marrow and reticulocytes in peripheral blood. In addition, the increase in micronuclei occurred at higher dose levels than the induction of AHF in L-E rats, suggesting that these phenomena are not interrelated. Therefore, the slight induction of micronuclei does not necessarily represent a specific genotoxic effect but may rather be secondary to other toxic effects. It should still be noted that contrary to all of the negative genotoxicity data, there are some studies from one laboratory reporting dose-dependent induction of micronuclei and sister chromatid exchanges in human lymphocytes treated with relatively low concentrations of TCDD in vitro (51–53).

Unfortunately, inaccurate reporting and the fact that the frequencies of micronuclei also in TCDD-treated lymphocytes were well within the normal baseline frequency (54) render the significance of these findings questionable.

Development of hepatocellular neoplasms and AHF appear to be correlated with hepatotoxicity in TCDD-treated rats (9, 10, 14, 26). Our data provide further support for this concept: (a) increased plasma ASAT, ALAT, and GGT activities were observed at the same dose levels that induced AHF, showing significant correlations; and (b) the same (≈100-fold) sensitivity difference between L-E and H/W rats was observed for indicators of hepatotoxicity and induction of foci.

Dose responses for induction of liver EROD activity were similar in L-E and H/W rats. These results are in agreement with our previous data after a single dose of TCDD (30, 55) and confirm that the AHR-mediated induction of CYP1A1 activity is normal also in H/W rats. The results also clearly indicate that enzyme induction and tumor promotion are not interrelated: (a) they follow different dose responses, as reported earlier (10, 56); and (b) H/W rats are as sensitive as L-E rats to CYP1A1 induction but 100-fold more resistant to the induction of AHF.

Thymus atrophy is one of the characteristic end points of dioxin toxicity. A decrease in relative thymus weights was parallel in both strains indicating similar potency of TCDD for this effect. Nevertheless, the magnitude of the decrease was clearly smaller in H/W than in L-E rats. These findings are in accordance with our earlier observations after a single dose of TCDD (57, 58) and indicate that thymus atrophy, which is an AHR-mediated phenomenon (15), develops also in H/W rats despite their deviant AHR. Increased relative liver weight is also a typical response elicited by TCDD and reflects enzyme induction-associated proliferation of endoplasmic reticulum in liver and a decreased amount of adipose tissue at higher dose levels. L-E rats were ~10-fold more sensitive, mainly because of more severe body weight loss.

In conclusion, a remarkable sensitivity difference of two orders of magnitude in induction of AHF by TCDD was found between L-E and H/W rats. The exceptional resistance of H/W rats is associated with an altered transactivation domain of the AHR. A similar sensitivity difference was observed in hepatotoxicity and long-term mortality, whereas the two strains were equally sensitive to AHR-mediated CYP1A1 induction and thymic atrophy. Our results imply that genetic differences may account for significant interindividual/intraspecies sensitivity differences in dioxin-induced carcinogenesis. Understanding the role of the AHR transactivation domain in carcinogenesis is therefore expected to result in improved risk assessment of dioxins.

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Liver Tumor-promoting Activity of 2,3,7,8-Tetrachlorodibenzo-\(p\) -dioxin (TCDD) in TCDD-sensitive and TCDD-resistant Rat Strains

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