Quantitative Analysis of Dose- and Time-Dependent Promotion of Four Phenotypes of Altered Hepatic Foci by 2,3,7,8-Tetrachlorodibenzo-p-dioxin in Female Sprague-Dawley Rats

Justin G. Teeguarden,*† Yvonne P. Dragan,* Jodi Singh,* Jennifer Vaughan,* Y.-H. Xu,* Thomas Goldworthy,‡ and Henry C. Pitot*†‡

*McArdle Laboratory for Cancer Research and the Medical School, University of Wisconsin, Madison, Wisconsin 53706–1599; †The Environmental Toxicology Center, B157 Steenbock, 550 Babcock Drive, University of Wisconsin-Madison, Madison, Wisconsin 53706; and ‡Integrated Laboratory Systems, Inc., P.O. Box 13501, Research Triangle Park, North Carolina 27709

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Determining both the mechanism by which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) acts as a tumor promoter and the shape of the dose-response curve at low doses remains an important goal of risk-assessment-directed research. In this report, we extend previous mechanistic and descriptive work done on the effect of TCDD on promotion in the two-stage model of hepatocarcinogenesis, to include lower, more clinically relevant doses. After initiation [PH + 10 mg diethylaminoarylamine (DEN)/kg], groups of female Sprague-Dawley rats were administered TCDD in one of four doses: 0.01, 0.1, 1.0, or 10 ng/kg/day for 1, 3, or 6 months. Early increases in liver weight (19–69%) due to hepatocyte hypertrophy were resolved after 3- or 6-month exposures to TCDD, and were not associated with the effects of TCDD on promotion. Non-focal cell proliferation in DEN-treated groups was significantly reduced after 1 or 3 months of exposure to 0.1 ng/kg/day TCDD, leading to U-shaped dose-response curves. TCDD effects on non-focal cell proliferation were not associated with effects on promotion. GSTP-positive AHF represented ~97% of the total AHF. Significant increases in both the volume fraction and the number of altered hepatic foci (AHF) were observed at the highest dose (10 ng/kg/day) for GSTP-positive AHF in DEN-treated groups. Increases in the number of G6Pase- and ATPase-deficient AHF/cm³ were observed at TCDD doses as low as 0.01 ng/kg/day. This is the lowest tumor-promoting dose of TCDD reported to date. This study represents an unusually complete data set for further dose-response analysis and simulation or mathematical modeling of TCDD-mediated promotion in the rat liver.

Key Words: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); promotion; altered hepatic foci; Sprague-Dawley rats; liver; dose response; glutathione s-transferase (GSTP); γ-glutamyl transpeptidase (GGT); canalicular adenosine triphosphatase (ATPase); glucose-6-phosphatase (G6P); cell proliferation; carcinogens; non-genotoxic.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most carcinogenic member of the polychlorinated dibenzo-p-dioxin (PCDD) family of compounds. Continual regulatory controversy surrounding the toxicity of these compounds has resulted in an unusually comprehensive characterization of their relative acute and chronic toxicity (Lucier et al., 1993; Poland, 1982a; Poland and Glover, 1979; Poland and Knutson, 1982b; Saadat et al., 1994; Safe, 1986; Waern et al., 1991). The most potent and widely studied member, TCDD, is present in low levels (0.1 to 0.3 pg/kg/day) in the human diet as a result of its inadvertent formation and release during combustion processes, including waste incineration and the manufacture of both paper and the herbicide 2,4,5-trichlorophenoxy-acetic acid.

TCDD is a non-genotoxic (Kociba and Schwetz, 1982; Poland and Glover, 1979; Randerath et al., 1988, 1990; Wassom et al., 1978), multi-site carcinogen when administered chronically at doses associated with liver toxicity in the female rat (Keenan et al., 1991; Kociba et al., 1978). At lower, more environmentally relevant doses, TCDD functions as a tumor promoter in the rat liver and mouse skin multistage models of carcinogenesis (Dragan et al., 1992; Flodstrom et al., 1991; Hemming et al., 1995; Maronpot et al., 1993; Pitot et al., 1980; Poland and Knutson, 1982b; Schrenk et al., 1994a; Sills et al., 1994; Stinchcombe et al., 1995; Tritsch et al., 1995, 1992; Waern et al., 1991). TCDD selectively promotes the clonal outgrowth of several populations of initiated cells by increasing the net growth rate through one or more epigenetic mechanisms. These include reduction of cell proliferation in normal hepatocytes (Bauman et al., 1995), reduction of intrafocal apoptosis (Schulte-Hermann et al., 1992; Stinchcombe et al., 1995), increased intrafocal cell proliferation (Buchmann et al., 1994), or some combination of these mechanisms.

The molecular mechanisms through which TCDD modulates these processes are currently unknown, but appear to be estrogen-dependent in the rat liver (Lucier et al., 1991; Sewall et al., 1993) and involve interaction with the aryl hydrocarbon.
receptor (AhR): aryl hydrocarbon nuclear translocator complex (Arnt). The effect of TCDD on hepatocyte cell proliferation, though clearly dose- and model-dependent, similarly remains uncertain; increases, decreases, and no change in non-focal cell proliferation (Fox et al., 1993; Lucier et al., 1991; Walker et al., 1998), as well as increases or no change in focal cell proliferation (Buchmann et al., 1994; Stinchcombe et al., 1995), have been reported.

Many studies have been completed in the two-stage hepatocarcinogenesis model examining the role of cell proliferation, apoptosis, protooncogene expression and activation, and alterations in growth factors (Dragan et al., 1992; Flodstrom et al., 1991; Hemming et al., 1995; Lucier et al., 1991; Maronpot et al., 1993; Pitot et al., 1980; Poland and Knutson, 1982b; Schrenk et al., 1994a,b; Sills et al., 1994; Stinchcombe et al., 1995; Tritscher et al., 1995, 1992; Waern et al., 1991) in an effort to determine the mechanism of action for tumor promotion and hence the critical steps that influence TCDD dose response. Many of these studies utilized necrogenic doses of DEN or examined only single high doses of TCDD or single exposure times. No studies have been completed that evaluate promotion-related endpoints at multiple doses and multiple exposure times, or at doses less than 0.1 ng/kg/day.

Initiation-promotion models of hepatocarcinogenesis utilizing necrogenic doses of DEN are widely used, useful tools for studying promotion. However, the relevancy of these studies to human risk estimation must be examined in the light of their limitations, which include, most importantly, the inability to separate the stage of promotion from the stage of progression (Scherer and Emmelot, 1975). Caution must be observed, especially with reference to tumor incidence, when interpreting the results of initiation-promotion studies utilizing necrogenic doses of DEN, which can lead to the development of HCC without promotion (Scherer and Emmelot, 1975).

In order to more accurately describe both the shape of the TCDD dose-response curve, the effects of TCDD on tumor promotion and cell proliferation should be quantified at lower doses and at multiple time points. In addition, re-evaluation of the effects of TCDD on tumor promotion and cell proliferation in the 2-stage model of hepatocarcinogenesis, with a non-necrogenic dose of DEN, is important, because this paradigm allows the clear separation between the stages of promotion and progression. The resulting data set on the time- and TCDD-dose-dependent promotion of AHF would be immediately useful for the testing and development of biologically motivated dose-response models.

The objectives of the current study are: (1) to investigate the dose-response relationship of TCDD-mediated tumor promotion in the 2-stage model of rat hepatocarcinogenesis at 1, 3, and 6 months; (2) to determine the shape of the dose-response curve at doses lower than those previously tested; (3) to determine the effect of TCDD dose and time of exposure on cell proliferation in non-focal hepatocytes; and (4) to simultaneously develop an appropriate data set for biomathematical-simulation modeling of the growth process of preneoplastic foci under the influence of TCDD.

**MATERIALS AND METHODS**

**Animals and treatments.** The experimental design describing the dose regimen, treatment groups, and timeline of the initiation-promotion study (Fig. 1) is similar to previously described protocols (Pitot et al., 1980). Female Sprague-Dawley rats weighing 150–200 g (Harlan Sprague-Dawley Co., Madison WI) were randomly assigned to one of 30 groups of 10 animals. A partial hepatectomy (PH) was performed on the animals. A subsequent study of liver weight before and after PH, after DEN dosing, and after 1 week recovery in Sprague-Dawley rats of the same weight revealed that PH removes 60% of the liver of rats in this weight class. The animals were administered a non-necrogenic, subcarcinogenic dose of the carcinogen diethylnitrosamine (10 mg DEN/kg, ig, Sigma Corp. St. Louis, MO) 24 h after the 60% PH, or the necrogenic, subcarcinogenic dose of the carcinogen diethylnitrosamine (10 mg DEN/kg, ig, Sigma Corp. St. Louis, MO) 24 h after the 60% PH, or the tricaprylin vehicle (controls). TCDD (Radian Corporation, 99% purity) dissolved in tricaprylin/acetone (24:1) was administered to those groups receiving the promoting agent by biweekly intraperitoneal injections of 0.00014 µg/kg, 0.0014 µg/kg, 0.014 µg/kg, or 0.14 µg/kg for a total exposure period of 1, 3, or 6 months. These doses correspond to 0, 0.01, 0.1, 1.0, and 10 ng/kg/day. Animals were fed Teklad 4% M/R crude diet #70001 ad libitum and housed and cared for according to the University of Wisconsin animal care program guidelines in an AAALAC-accredited Animal Care Facility. Osmotic pumps (Alzet Corporation, Palo Alto, CA, 2 ml) containing 2.3 ml of a 20 mg/ml BovI solution were implanted subcutaneously, 1 week prior to sacrifice, to label replicating hepatocytes.

**Immunohistochemistry/stereology.** At sacrifice, 2-mm slices of each of the 3 remaining lobes of the liver were obtained, juxtaposed on filter paper, and immediately frozen as composite tissue blocks on dry ice. Two composite tissue blocks were prepared from each liver, and 4-10-µm thick serial sections were cut and stained successively for the placental isozyme of glutathione S-transferase (GSTP), γ-glutamyl transpeptidase (GGT), canalicular adenosine triphosphatase (ATPase) and glucose-6-phosphatase (G6P) by previously described methods (Dragan et al., 1992; Goldsworthy and Pitot, 1985; Hendrich and Pitot, 1987; Sato et al., 1984). Two representative samples from each animal were analyzed for the 4 phenotypes. The number, size, and phenotypic distribution of the AHF were determined by the methods of quantitative stereology as described by Campbell et al. (1982, 1986). The number of
labeled nuclei in non-focal cells was determined by examining the nuclei within 2 randomly selected fields of at least 1000 non-GSTP staining cells from each animal. A total of 18,000–20,000 nuclei were examined per dose group, and the percent of nuclei labeled with BrdU was reported.

Statistical analysis. There were large inter-rat and intra-rat variations in the number and volume fraction data that are characteristic of animal bioassay data. To obtain homogeneity of variance, the data were either log transformed ($\log_{10}(\text{AHF/cm}^3)$) or ArcSin(SQRT(Volume%)) transformed. Prior to log transformation, the value 0.1 AHF/cm$^3$ was substituted for observations of 0.0 AHF/cm$^3$. The data were analyzed by parametric ANOVA followed by Dunnett’s test. Cell proliferation data were analyzed by parametric ANOVA followed by Duncan’s multiple range test. Transformation of cell proliferation data did not result in homogeneity of variance, or normality.

RESULTS

Liver Weight

Relative liver weight calculated at each dose/time point, is presented in Figure 2. Dose-dependent changes in liver weight were observed in both the DEN and non-DEN-treated groups after a 1-month exposure to TCDD. The increase in liver weight was dramatic, showing a 58–69% increase in the 10 ng/kg/day groups. The relative liver weight was similarly increased 19-32% in the 1-ng/kg/day groups ($p < 0.05$ level Dunnett’s test). This is consistent with results from other rodent models, including both rats and mice (Birnbaum et al., 1990; Hebert et al., 1990; Maronpot et al., 1993; Schrenk et al., 1994a). The magnitude of the increase was greatest in non-initiated groups (69 vs. 58%, 32 vs. 19%). With the exception of a modest 12% increase in the 10 ng/kg/day DEN-treated group, after a 3-month exposure to TCDD, the increase in liver weight was completely resolved after a 3- or 6-month exposure to TCDD. Previous studies of promotion in the 2-stage model and higher doses of TCDD or other polychlorinated aromatic hydrocarbons have shown liver hypertrophy after 3–5 months’ promotion (Schrenk et al., 1994a; Waern et al., 1991).

Increased relative liver weight could be the result of hypertrophy and/or hyperplasia. Examination of the number of nuclei/unit area of tissue and direct measurement of relative hepatocyte size (data not shown) in control and high-dose groups indicated that the primary response in the liver was hepatocyte hypertrophy. Cell proliferation data obtained by BrdU labeling of replicating hepatocytes do not support a role for cell proliferation in the increase in relative liver weights (below). Animal body weights were not significantly affected by TCDD administration during the study period.

Non-focal Cell Proliferation

S-phase BrdU labeling of nuclei is a heavily utilized surrogate measure of cell proliferation in vivo (Fox et al., 1993; Goldsworthy et al., 1996; Maronpot et al., 1993; Stinchcombe et al., 1995). To prevent the confounding effect of TCDD-induced hepatocyte hyperplasia and hypertrophy (Buchmann et al., 1994) on estimating cell proliferation, the analysis was completed on a per nuclei basis.

TCDD treatment had no effect on the cell proliferation rate of non-focal hepatocytes at any dose or time point in the non-initiated rats compared with control rates (Fig. 3). This supports observations at higher TCDD doses from several other laboratories (Fox et al., 1993; Maronpot et al., 1993; Stinchcombe et al., 1995). One-month’s exposure to 10-ng/kg/day TCDD resulted in an increase in cell proliferation when compared with the 0.1 ng/kg/day dose group ($p < 0.05$, Duncan’s multiple-range test). Non-focal cell proliferation in DEN-initiated control and TCDD-treatment groups was generally greater than that of non-DEN-treated groups after 1, 3, or 6 months. This indicates a clear DEN effect on non-focal cell proliferation in the rat liver. In DEN-treated animals, one month of exposure to either 0.1 or 1 ng/kg/day TCDD caused an attenuation of cell proliferation when compared with either
the control or 0.01 ng/kg/day dose group ($p < 0.05$, Duncan’s multiple range test) (Fig. 3). These doses are lower by a factor of 3–30 than those previously tested. A reduction in hepatocyte proliferation was also observed at 3 months in the 0.1 ng/kg-day group. The resulting dose-response curves were U-shaped, similar to those seen in previous studies of tumor formation and cell proliferation (Kociba et al., 1978; Maronpot et al., 1993). These effects were not observed in the 6-month treatment group. The loss of the TCDD-dependent attenuation of cell proliferation at this time point may be the result of higher hepatic concentrations of TCDD reached after a 6-month administration of 0.1 ng/kg/day TCDD.

The observed increases and decreases in non-focal cell proliferation were not consistently observed at all 3 time points. This lack of consistent changes at promoting doses of TCDD suggests a lack of correlation between changes in non-focal cell proliferation and hepatic tumor-promoting activity.

### Hepatic Promotion: Phenotypic and Size Class Distribution

The number and size class of 4 biochemical phenotypes of AHF were collected: GSTP-positive, GGT-positive, ATPase-deficient, and G6Pase-deficient. The phenotypic distribution of AHF was dominated by those foci staining positively for GSTP. In all groups including DEN/saline controls, 78–99% (average 97%, SEM 2.3%) of the foci were GSTP-positive, the value being greater than 90% in all but one group. The focal volume fraction was similarly dominated by GSTP-positive AHF, with an average of 89% (SEM, 1.1%) of the total volume being GSTP-positive cells. Others have noted the value of this marker for hepatic, preneoplastic lesions (Cameron et al., 1978; Dragan et al., 1992; Sato et al., 1984), and it has been used extensively to study TCDD- and phenobarbital-dependent promotion (Dragan et al., 1991a, 1993a, 1992, 1991b, 1993b; Maronpot et al., 1993; Schrenk et al., 1994a). Possible dose- or time-dependent shifts in the fraction of lesions staining for GSTP have not been explored. Large dose- or time-dependent shifts in the fraction of total lesions staining positive for GSTP would influence the interpretation of any promotion study completed with a single marker such as GSTP. This study clearly shows that dose and time did not have a noticeable or consistent effect on the fraction of foci or the fraction of the total volume percent of liver occupied by GSTP-positive AHF. Such consistency is necessary for this marker to be used for accurate measurement of the dose-response characteristics of TCDD and other promoting agents.

Data on size class distributions are rich in information and provide a more detailed analysis of the influence of time and dose on the growth of preneoplastic lesions than volume fraction and AHF multiplicity data alone. Saltykov size class data for each of the 4 markers were collected for each dose and time point in the study. The size of focal transections will have some degree of correlation with focal volume fraction, which shows an increasing trend with TCDD dose. Analysis of size class distributions was carried out only in the DEN-treated groups. Interestingly, a strict dependence of the size of focal transections on dose was not observed (Fig. 4). After 1- and 6-month exposures to TCDD, only the highest dose displayed an increase in the size class of GSTP-positive AHF. Three-month exposure resulted in equivalent size class distributions in the control and high-dose groups, though there was an increase in volume fraction at this time point. Dose was not related to increased size class in the remaining dose groups. Portier et al.
(1996) also reported no change in median size class for TCDD doses between 3.5 and 35.7 ng/kg/day, a dose range larger than that examined in the current study. They reported ~25% increase in the size of the median-size class only at the high TCDD dose, 125 ng/kg/day. Dose-dependent changes in the median size class of GGT-positive AHF in 1,2,3,7,8-pentachlo-
rodibenzo-\textit{p}-dioxin (PeCDD)-, 2,3,4,7,8-pentachlorodibenzo-furan (PeCDF)-, and TCDD-treated rats were reported by Waern et al. (1991). The increase was modest, less than ~25% between control and the high-TCDD dose (125 ng/kg/day), and was similarly small for the PeCDD- and PeCDF-treated groups.

Time, however, had a much stronger effect on size class distributions than dose (Fig. 4). In contrast to dose effects, the median diameter of the focal transections increased as a function of time at all doses of TCDD and in the control group. Time had a similar effect on the percentage of the total volume fraction of the liver occupied by the largest size classes of GSTP-positive AHF. After a 1-month exposure to TCDD, the smallest 80% of the GSTP lesions account for between 46% and 58% of the total volume fraction. This value decreases to 30-42% of the volume fraction after 6 months of exposure to TCDD. At this point, the largest 20% of the lesions account for more than 60% of the total volume fraction of GSTP AHF (Fig. 5). As the time of exposure increases, it appears that a decreasing fraction of AHF accounts for the majority of the total volume fraction. This may represent the expansion and eventual dominance of a sub-population of AHF with a greater growth advantage.

\textbf{Hepatic Promotion: Effect of TCDD on the Number and Volume Fraction of AHF}

As a measure of promotion, the number and volume fraction of AHF were determined for each dose and time point. These data are presented in Figures 6 and 7. Post-initiation administration of 10-ng/kg/day TCDD for 1, 3, or 6 months led to a dramatic increase (from 1.6- to 6-fold) in the total number of GSTP-positive AHF in the liver (Fig. 6). The number of GSTP-positive AHF was also increased 2.5-fold after a 6-month exposure to 1-ng/kg/day TCDD. GGT-positive AHF was similarly increased 1.8- to 4.5-fold after a 1- or 3-month exposure to the highest dose of TCDD (Table 1). Although ATPase- and G6Pase-negative lesions were a minor component of the total population of AHF, their numbers were increased (16- to 340-fold) at all doses of TCDD after a 1-month exposure (Table 1). This is the first report of promoting doses of TCDD below 1 ng/kg/day.

With the exception of GSTP-positive AHF in the highest dose group, TCDD did not cause an increase in the numbers of putatively preneoplastic AHF of any phenotype at dose or exposure duration in non-initiated animals (data not shown). These results suggest that prior generation of putative preneoplastic lesions is requisite for TCDD action as a promoter, especially when measured at time points as early as 1 to 6 months.

Treatment of non-initiated animals with 0.1 ng/kg/day resulted in lower total numbers of AHF when compared with other dose groups (\( p < 0.05 \), Tukey-Kramer HSD) (data not shown). The resulting dose-response curve was U-shaped. However, very large standard errors in these data and the lack of consistent U-shaped dose-response curves in this study suggest that this observation may be the result of a limited sampling of mean values.

Because volume fraction reflects increases in both the number and size of AHF, it is considered a more accurate measure of promotion (Pitot, 1990). With one exception, in initiated animals, high-dose treatment (10 ng/kg/day) resulted in an increase in the volume fraction of the liver occupied by AHF of all 4 phenotypes after 1-, 3-, or 6-month exposure (\( p < 0.05 \), Dunnett’s test) (Table 2, Fig. 7). The volume fraction of the dominant marker, GSTP, accounting for 89% of the volume fraction, was elevated in only the 10 ng/kg/day dose group (Fig. 7). TCDD-dependent promotion was less consistent across phenotypes at lower doses. One-month exposure to 1 ng/kg/day TCDD led to increases in the volume fraction of only ATPase- and G6Pase-deficient AHF. The lowest promot-

\begin{figure}[h]
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\caption{The effect of TCDD dose on the percentage of total AHF responsible for a given fraction of the total volume of GSTP-positive AHF in DEN-initiated rats treated with TCDD for 1, 3, or 6 months. Horizontal lines indicate the percentage of the total volume fraction attributable to the smallest 80% of GSTP-positive AHF.}
\end{figure}
ing dose of TCDD was 0.1 ng/kg/day (p<.05, Dunnetts test), observed in the 1-month exposure group. At this dose, the volume fraction of G6Pase-deficient AHF was increased 8-fold.

TCDD DISCUSSION

Previous investigations designed to examine the dose response for tumor promotion and to determine the molecular mechanisms responsible for TCDD-dependent promotion of AHF have utilized either necrogenic doses of DEN for initiation, or evaluated either single time points, or single doses of TCDD (Dragan et al., 1992; Flodstrom et al., 1991; Hemming et al., 1995; Lucier et al., 1991; Maronpot et al., 1993; Pitot et al., 1980, 1987; Schrenk et al., 1994a,b; Sills et al., 1994; Stinchcombe et al., 1995; Tritscher et al., 1995, 1992; Waern et al., 1991). While effective to the degree that these protocols lead to large responses, the utility of these experiments for studying tumor promotion and clearly defining the shape of the dose-response curve is limited by the experimental conditions. The present study was designed to re-evaluate the TCDD dose response for hepatic tumor promotion, cell proliferation, and apoptosis after a non-necrogenic dose of DEN, and to extend previous efforts by examining multiple non-hepatotoxic doses of TCDD at early and intermediate time points. A principal goal of this work was to establish a complete data set for simulation and dose-response modeling across time and dose.

Though hepatic initiation-promotion models that utilize necrogenic (175–200 mg/kg) and non-necrogenic (10 mg/kg) doses of DEN each have distinct advantages, interpretation of results from these models should include a consideration of the differences in the biology of each model. Liver regeneration after a necrogenic dose of DEN occurs through the activation and proliferation of both parenchymal cells and epithelial progenitor cells, termed “oval cells” (Solt et al., 1977), which are capable of differentiating into hepatocytes, intestinal glandular epithelium, or pancreatic-like tissue (Debeva et al., 1995). In contrast, oval cell proliferation is not observed after partial hepatectomy, even when treatment includes administration of 10 mg/kg of DEN (HCP, unpublished observations). Although oval proliferation in the necrogenic and non-necrogenic models of initiation has not been evaluated in identical subjects and experimental conditions, it appears that because they may expose two developmentally dissimilar populations of cells to DEN, the target cell populations for initiation and promotion may be different. In addition, the stage of promotion cannot be clearly separated from progression when doses of DEN that are both necrogenic and carcinogenic are employed (Dragan et al., 1994). In light of this evidence, a rat model utilizing non-necrogenic doses of DEN may be more appropriate for generation of data for bio-mathematical modeling of the promotion process and for comparative risk estimation.

This study extends previous work by examining much lower TCDD doses and multiple time points for evidence of promotion. Previous studies of TCDD-dependent promotion have consistently reported effects at dose levels between 10 and 200 ng/kg/day; the lowest reported promoting dose is 6.3 ng/kg/day (Waern et al., 1991) for GGT-positive AHF. In addition to the expected promotion of GSTP-positive AHF at the highest dose,
we report a transient increase (16–340 fold) in the number of G6Pase- and ATPase-deficient AHF after administration of $0.01\text{ ng/kg/day}$ TCDD for one month. This further extends work completed in this and other laboratories (Buchmann et al., 1994; Dragan et al., 1992; Flodstrom et al., 1991; Hemming et al., 1995; Lucier et al., 1991; Maronpot et al., 1993;)

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<td>693.0–283.2</td>
<td>113.5</td>
<td>177.0–72.8</td>
<td>168.3</td>
<td>291.0–97.2</td>
</tr>
</tbody>
</table>

Note. Values are % of the liver occupied by AHF of one of the four phenotypes, followed by the 95% confidence limits (CL). To obtain homogeneity of variance, values were $\text{ArcSin(SQRT(%))}$ transformed before statistical analysis. Values were compared with control with an ANOVA followed by Dunnett’s test, with a significance level of $p < 0.05$. Only DEN-treated groups are presented. Phenotypes analyzed include gamma-glutamyl transpeptidase (GGT), canalicular adenosine triphosphatase (ATPase), and glucose-6-phosphatase (G6P).

* Significantly different from control values.

we report a transient increase (16–340 fold) in the number of G6Pase- and ATPase-deficient AHF after administration of $\geq 0.01\text{ ng/kg/day}$ TCDD for one month. This further extends work completed in this and other laboratories (Buchmann et al., 1994; Dragan et al., 1992; Flodstrom et al., 1991; Hemming et al., 1995; Lucier et al., 1991; Maronpot et al., 1993;)

### TABLE 2

The Number of Altered Hepatic Foci/cm$^3$

<table>
<thead>
<tr>
<th>TCDD dose (ng/kg/day)</th>
<th>Exposure duration (months)</th>
<th>ATP Upper 95% CL</th>
<th>Lower 95% CL</th>
<th>G6P Upper 95% CL</th>
<th>Lower 95% CL</th>
<th>GGT Upper 95% CL</th>
<th>Lower 95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1</td>
<td>0.01</td>
<td>0.02–0.00</td>
<td>0.00</td>
<td>0.01–0.00</td>
<td>0.03</td>
<td>0.05–0.02</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.14</td>
<td>0.23–0.06</td>
<td>0.06</td>
<td>0.11–0.03</td>
<td>0.09</td>
<td>0.16–0.04</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.38</td>
<td>0.51–0.27</td>
<td>0.22</td>
<td>0.33–0.13</td>
<td>0.15</td>
<td>0.21–0.10</td>
</tr>
<tr>
<td>0.01</td>
<td>1</td>
<td>0.04</td>
<td>0.07–0.02</td>
<td>0.04</td>
<td>0.05–0.02</td>
<td>0.02</td>
<td>0.03–0.02</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.09</td>
<td>0.13–0.05</td>
<td>0.07</td>
<td>0.10–0.04</td>
<td>0.08</td>
<td>0.12–0.05</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.70</td>
<td>0.85–0.57</td>
<td>0.32</td>
<td>0.42–0.24</td>
<td>0.21</td>
<td>0.31–0.13</td>
</tr>
<tr>
<td>0.10</td>
<td>1</td>
<td>0.04</td>
<td>0.07–0.02</td>
<td>0.02*</td>
<td>0.03–0.01</td>
<td>0.02</td>
<td>0.04–0.01</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.12</td>
<td>0.21–0.06</td>
<td>0.09</td>
<td>0.16–0.04</td>
<td>0.13</td>
<td>0.19–0.08</td>
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<td></td>
<td>6</td>
<td>0.55</td>
<td>0.86–0.31</td>
<td>0.20</td>
<td>0.33–0.10</td>
<td>0.23</td>
<td>0.36–0.13</td>
</tr>
<tr>
<td>1.00</td>
<td>1</td>
<td>0.06*</td>
<td>0.10–0.03</td>
<td>0.04*</td>
<td>0.05–0.03</td>
<td>0.03</td>
<td>0.07–0.01</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.24</td>
<td>0.40–0.12</td>
<td>0.10</td>
<td>0.17–0.04</td>
<td>0.18</td>
<td>0.27–0.11</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.79</td>
<td>1.20–0.48</td>
<td>0.35</td>
<td>0.58–0.17</td>
<td>0.38</td>
<td>0.68–0.17</td>
</tr>
<tr>
<td>10.00</td>
<td>1</td>
<td>0.55*</td>
<td>0.77–0.37</td>
<td>0.28*</td>
<td>0.42–0.16</td>
<td>0.19*</td>
<td>0.30–0.11</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.53*</td>
<td>0.99–0.21</td>
<td>0.22*</td>
<td>0.44–0.09</td>
<td>0.23</td>
<td>0.43–0.09</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2.60*</td>
<td>4.40–1.25</td>
<td>0.85*</td>
<td>1.40–0.46</td>
<td>0.79*</td>
<td>1.30–0.39</td>
</tr>
</tbody>
</table>

Note. Values are number of AHF/cm$^3$ of one of the four phenotypes analyzed, followed by the 95% confidence limits (CL). In order to obtain homogeneity of variance, values were Log$_{10}$ transformed before statistical analysis. Values were compared with control with an ANOVA followed by Dunnett’s test, with a significance level of $p < 0.05$. Only DEN-treated groups are presented. Phenotypes analyzed include gamma-glutamyl transpeptidase (GGT), canalicular adenosine triphosphatase (ATPase), and glucose-6-phosphatase (G6P).

* Significantly different from control values.
at lower doses, for multiple markers, and time points. Although these changes were statistically significant, caution should be used before interpreting these results as evidence of a low threshold for promotion. The results were dependent on several "0" observations in the 9-10 animal control groups. The true values for these animals are unlikely to be zero; the AHF present were probably below the size limit of detection for this study (foci $\leq 125 \mu m$ in diameter). This is substantiated by the disappearance of zero observations at later time points where AHF in control groups were larger and thus observable. Consistent increases in the numbers of AHF/cm$^3$ were observed only after 6-month exposures to 10 ng/kg/day TCDD, a time point where zero observations did not affect control values.

Confirming previous reports, promotion, as measured by an increase in volume fraction, was observed at all time points and phenotypes of AHF only in the high-dose groups. The lowest dose causing an increase in the volume fraction was 0.1-ng/kg/day TCDD. Observations of zero AHF also influenced this result. The U-shaped dose response reported earlier for tumor incidence (Kociba et al., 1978) was not observed. The clear threshold reported here for the dominant phenotype of AHF confirms other reports (Maronpot et al., 1993; Pitot et al., 1987) and indicates that promotion in response to TCDD is not measurably linear at low doses. This classic characteristic of tumor-promoting agents is not accounted for in the current regulations based on TCDD cancer potency estimates.

The lack of significant increases in the number of AHF/cm$^3$ at any dose or time point, in the absence of initiation with DEN, does not support reports that TCDD may have mutagenic activity (Moolgavkar et al., 1996; Portier et al., 1996). Although no direct measurements of mutagenicity were made, it is safe to conclude, at this level of analysis, that any putative mutagenic activity TCDD has is small in comparison to that induced by low doses of DEN. Perhaps more importantly, any mutagenic activity is far less important to the development of AHF than the promoting effects of TCDD.

While biochemical phenotypes are used to identify AHF, the underlying behavioral phenotypes, which could be determined by classifying growth characteristics, remain unknown. The size class analysis of GSTP-positive AHF demonstrates that, after 6 months but not after a 1-month exposure to TCDD, a relatively small group of AHF (20%) account for greater than 60% of the total volume fraction of GSTP AHF. One interpretation of this observation is that a relatively small group of rapidly growing lesions at late time points is responsible for the majority of the increases in volume fraction. Alternatively, this may result from a combination of a strong influence of time on size class and the stochastic nature of clonal growth. Because clonal growth is exponential (at least when clones are relatively small), rapid early growth that reflects random chance (stochasticity) will result in one, or at most a very few, clones becoming much larger than the general population of AHF. These will eventually account for a disproportionate amount of the volume fraction (Rory Conolly, personal communication,

**FIG. 7.** The volume fraction of the liver occupied by GSTP-positive AHF in DEN-initiated and non-initiated TCDD-treated rats. Rats were administered TCDD every 2 weeks for 1, 3, or 6 months. Data points are mean values, and bars represent 95% confidence intervals; *indicates values statistically different from controls ($p<0.05$, Dunnett’s test).
This population of AHF may represent a unique behavioral phenotype. If biochemical analysis is combined with focal cell proliferation and apoptosis rates, it may permit the identification of that and other behavioral phenotypes. Research focusing on behavioral rather than biochemical phenotypes may provide new insights into the mechanisms of tumor promotion.

With the exception of two dose groups, non-focal cell proliferation measured at the 1-month time point was elevated compared with 3- and 6-month time points. This effect was also observed in control groups, leading to the conclusion that the 1-month increase is primarily a response to DEN administration or a residual effect of the partial hepatectomy. Hepatic levels of TCDD, established after 6 months of administration of TCDD at 0.01 to 10 ng/kg/day, had no effect on non-focal cell proliferation. Lower, non-steady-state levels at 1 and 3 months (0.1 to 1 ng/kg/day) reduced non-focal cell proliferation and resulted in a U-shaped dose-response curve. This report of a dip in the TCDD dose-response curve for non-focal cell proliferation may result from TCDD-mediated mito-inhibition of one or more populations of hepatocytes. Such populations could include non-focal normal cells, or non-GSTP-positive focal cells, whose cell proliferation is inhibited by TCDD treatment. Dose-dependent reductions in cell proliferation are absent in the 6-month treatment groups. The disappearance of this effect may be the result of the eventual extinction of these mito-suppressed hepatocytes. This is consistent with the hypothesis that TCDD-dependent promotion is the result of focal cells escaping from TCDDD-induced mito-suppression (Mills and Andersen, 1993) and supported by the lack of this effect in the non-DEN-treated animals. Alternatively, the attenuation of non-focal cell proliferation may be a very low-dose effect observed only at early time points where hepatic concentrations are lower than equilibrium concentrations thought to be reached at 6 months (Tritscher et al., 1992).

It is evident from this report and others that, in the initiation-promotion model, increase in non-focal cell proliferation by TCDD occurs in female rats only after chronic exposure to a 100–125 ng/kg/day dose of TCDD, which is both hepatotoxic and tumorigenic (Kociba et al., 1978; Tritscher et al., 1995), for 30 weeks (Lucier et al., 1991; Maronpot et al., 1993; Tritscher et al., 1995). The effect is ovarian hormone-dependent (Lucier et al., 1991). In the present study, increases in non-focal cell proliferation are not observed at lower doses of TCDD (0.1–10 ng/kg/day) where promotion of AHF is evident. Others have also reported a lack of a consistent effect of TCDD on non-focal cell proliferation (Maronpot et al., 1993; Sewall et al., 1993). This indicates that alteration of non-focal cell proliferation is not directly involved in the TCDD-mediated mechanism of promotion. Similarly, dose-dependent increases in focal-cell proliferation have not been demonstrated. Currently, the leading hypothesis of the mechanism of action of TCDD as a promoter is focal cell-specific reductions in apoptosis that lead to increases in the net growth rate of AHF (Stinchcombe et al., 1995). Data collected on focal cell apoptosis rates in this study were inconclusive. The AHF were sufficiently small as to make counting apoptosis and cell proliferation inaccurate and virtually unfeasible.

Hepatic hypertrophy is a common response to chemicals that induce drug-metabolizing enzymes including promoting agents such as peroxisome proliferators, TCDD and PCBs (Jian et al., 1997). This Ah receptor-dependent effect (Birnbaum et al., 1990) has been consistently reported in several species and model systems (Hebert et al., 1990; Maronpot et al., 1993; Schenk et al., 1994a; Waern et al., 1991). Enlargement of the cytosolic compartment, specifically, a large increase in the total amount of smooth endoplasmic reticulum, is primarily responsible for liver hypertrophy after treatment with PCBs (Jian et al., 1997), and this is probably true for TCDD treatment as well. Though hypertrophy appears to be a common response in rodents, one study in female rhesus monkeys administered daily doses of 80 μg of Aroclor 1254 for 6 years showed a 50% increase in liver weight attributable to hyperplasia (Arnold et al., 1997). This unique, long-term primate study, showing hepatic hyperplasia rather than hypertrophy, raises a question about the similarity of response between rodents and humans, especially given the consistency of the effect in rodent models. In this study, we report hepatocyte hypertrophy after administration of 1 or 10-ng TCDD/kg/day for one month. Studies of gene expression or other molecular responses during this period or after acute administration of TCDD may be influenced by alterations responsible for, or resulting from, hypertrophy or the large changes in smooth ER. It has been suggested that liver hypertrophy is frequently associated with promoting activity of chemicals. In this report, we show that hypertrophy is disassociated from promotion. In fact, since the response appears to be resolved after 3- or 6-month administration of TCDD, a period during which promotion is clearly still occurring, it may be appropriate to delay mechanistic studies of promotion in the liver until these later time points.

Two-stage models, a mathematical description of the carcinogenic process, are widely used as a paradigm for modeling carcinogenesis and, with increasing frequency, for the analysis of the carcinogenic properties of chemicals of regulatory concern (Sherman and Portier, 1997). These constructs have been previously used to successfully model the spontaneous (Conolly and Kimbell, 1994) and TCDD-mediated growth (Conolly and Andersen, 1997; Moolgavkar et al., 1996; Portier et al., 1996) of AHF. Review of several previous efforts clearly indicates that the parameter estimates used to fit a single time period will lead to unrealistically high volume fractions at later time points (Conolly and Andersen, 1997; Portier et al., 1996). More complete data sets such as the one developed in this study, with number and volume-fraction data as a function of both time and dose, will provide a more rigorous test of the ability of these models to describe the behavior of the AHF under the influence of TCDD, and will reproduce the observed dose-response curves. Data from this study are currently being
used to test stochastic clonal growth models of TCDD-mediated promotion.

Our understanding of the mechanism of action for promoting agents remains incomplete. Studies of the mechanics of these promoting agents—phenobarbital, estrogen, peroxisome proliferators, and PCBs—indicate that increase in hepatic cell proliferation is an effect common to these compounds (Cattley et al., 1996; Deml and Oesterle, 1982; Luebeck et al., 1991; Wysnner et al., 1996; Wölfe et al., 1988; Yager et al., 1984). Change in cell proliferation rates has been implicated as a mechanism of action for these non-genotoxic carcinogens (Moolgavkar and Luebeck, 1992). Here we show that changes in non-focal cell proliferation are not consistently altered in a dose-dependent fashion and are unlikely to be directly related to the promoting effects of TCDD. Future studies should be designed to generate AHF of sufficient size and number to allow determination of intrafocal apoptosis rates, which are reduced in a dose-dependent fashion in response to TCDD (Stinchcombe et al., 1995). For increases in volume fraction of AHF, there was a clear threshold for the promoting effects of TCDD on GGT-positive and GSTP-positive AHF of 1.0 ng/kg/day. These two markers accounted for the majority of AHF in this study. The threshold for increases in volume fraction of G6Pase-deficient AHF was 0.01 ng/kg/day in the 1-month exposure group and 1.0 ng/kg/day in the 3- and 6-month exposure groups. Consistent effects on promotion for these markers were not observed below the 10 ng/kg/day dose-group. Evidence that the response in rats does not proceed linearly through zero dose/zero response should be carefully considered when risk determinations are made for TCDD. With the exception of transient increases in G6Pase-deficient and ATPase-deficient AHF after 1-month exposures to ≥0.01 ng/kg/day TCDD, consistent increases in the number of AHF/cm³ were not observed below 10 ng/kg/day. Analysis of the dependence of time on size class suggests that the expansion and eventual dominance of a sub-population of AHF with a greater growth advantage may be an important element of promotion. Future studies should attempt to identify these AHF by combining biochemical classification (GSTP, GGT, G6Pase, ATPase) with measures of intrafocal apoptotic rates. Focusing on this highly responsive subset of AHF may provide clearer evidence for the effects of TCDD on the growth of these preneoplastic lesions. Although clear evidence for a mechanism of action did not emerge from this study, the incorporation of these data into stochastic clonal growth models (in progress) will facilitate a new analysis, which may lead to new descriptions of the behavior of these AHF at the tested doses.

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Promotion of preneoplastic foci in rat liver with 2,3,7,8-tetrachlorodibenzo-p-dioxin, 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin and a defined mixture of 49 polychlorinated dibenzo-p-dioxins. *Carcinogenesis* **15**, 509–515.


