TOXICOLOGICAL SCIENCES **66,** 54–61 (2002)
Copyright © 2002 by the Society of Toxicology

Nonadditive Hepatic Tumor Promoting Effects by a Mixture of Two Structurally Different Polychlorinated Biphenyls in Female Rat Livers

C. E. Dean, Jr.,*[,1] S. A. Benjamin,* L. S. Chubb,* J. D. Tessari,† and T. J. Keefe†

**Department of Pathology and †Department of Environmental Health, College of Veterinary Medicine and Biomedical Sciences, Center for Environmental Toxicology and Technology, Colorado State University, Fort Collins, Colorado 80523*

Received June 20, 2001; accepted November 9, 2001

This study evaluates and quantifies the interactive hepatic tumor promoting effects of two PCBs, the Ah receptor agonist PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl) and the constitutive androstane receptor (CAR) agonist PCB 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl). Promotion of altered hepatic foci was evaluated utilizing a medium-term 8-week bioassay for promoters of hepatocarcinogenesis. The assay employs placental glutathione-S-transferase positive (GST-P+) liver cell foci as markers of preneoplasia in female Fischer 344 rats treated with the known initiator diethylnitrosamine followed by partial hepatectomy and by gavage exposure to test chemicals. GST-P+ foci were quantified by histomorphometry and were reported as areas and numbers of GST-P+ foci within the area of liver examined. For PCB 126, the doses were 0.1, 1.0, and 10 μg/kg body weight. For PCB 153, the doses were 10, 100, 1000, 5000, and 10,000 μg/kg body weight. Combined PCB 126 and 153 exposures were 0.1 + 10, 1 + 100, 10 + 1000, 10 + 5000, and 10 + 10,000 μg/kg, respectively. Individual PCB treatment resulted in dose dependent increases in liver and adipose concentrations. Hepatic PCB 153 levels were significantly increased ($p < 0.01$) after combined exposure. Treatment with PCB 126 or PCB 153 alone resulted in a significant ($p < 0.01$) dose dependent increase in GST-P+ foci area and number compared with controls. Treatment with the mixture of PCB 126 and 153 resulted in antagonistic GST-P+ focus formation ($p < 0.001$) for both foci area and number. The less than additive effect was present at all 5 PCB 126/PCB 153 dose combinations, including the low doses of PCB 126 and 153 that did not show significant promotional activity alone.

Key Words: **carcinogenesis; polychlorinated biphenyls; liver; rat; promotion; altered hepatic foci; GST-P.**

To date, most toxicological and carcinogenesis data have been generated from studies performed on single chemicals. Yet in reality, humans are simultaneously and continuously exposed to a myriad of chemicals in their daily environment. A large number of agents capable of initiating and/or promoting carcinogenesis are recognized, many of which are present as mixtures at hazardous waste sites (Yang and Rauckman, 1987). For these reasons, it is important to understand the possible toxicologic interactions that can occur in chemical mixtures.

Polychlorinated biphenyls (PCBs) are halogenated aromatic hydrocarbons that have been used widely in industry and are now recognized as worldwide environmental pollutants (Safe, 1994). The biological effects induced by PCBs are dependent on the molecular structure of individual congeners (McFarland and Clarke, 1989; Safe, 1994). Congeners lacking or having one chlorine substitution in the ortho position with an additional chlorine in the para position of the biphenyl ring have a more co-planar conformation. PCB congeners substituted in both para positions and at least two meta positions are structurally related to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and they elicit comparable biological effects including binding with the aryl hydrocarbon receptor (AhR), induction of the monooxygenase enzymes cytochrome P450 1A1 and 1A2 (CYP1A1 and CYP1A2), thymic involution, the induction of a wasting syndrome, increased mortality, and a variety of toxic effects in the liver including carcinogenesis (Safe, 1990). PCB congeners, which bind the AhR and elicit the previously described effects, will be referred to as AhR agonist congeners in this text. Introduction of two or more chlorines in an ortho position results in decreased co-planarity between the two phenyl rings due to stearic interactions. Such PCB congeners exhibit liver tumor promoting activity that is "phenobarbital-like." These compounds are not as potent as the AhR agonist congeners but, like phenobarbital (PB), can induce hepatic tumor promotion. PCBs of this less planar group induce CYP2B1 and CYP2B2 or both CYP1A1/2 and CYP2B1/2 (Safe, 1994; Safe *et al.*, 1985). The induction of CYP2B1 by less planar PCBs congeners is mediated by the nuclear receptor constitutive androstane (CAR) (Muangmoonchai *et al.*, 2001) and will be referred to as CAR agonists in this text.

Human epidemiologic data on the carcinogenicity of PCBs have been equivocal. While no overall increases in cancer-related mortality could be correlated with occupational PCB exposure, some studies reported nonstatistically significant increases of specific cancers, including the grouping of liver, gall bladder, and biliary tract cancers (Kimbrough, 1995). Although

[1] To whom correspondence should be addressed at Colorado State University, Department of Pathology, 300 W. Lake Street, Fort Collins, CO 80523-1671. Fax: (970) 491-8304. E-mail: cdean@colostate.edu.

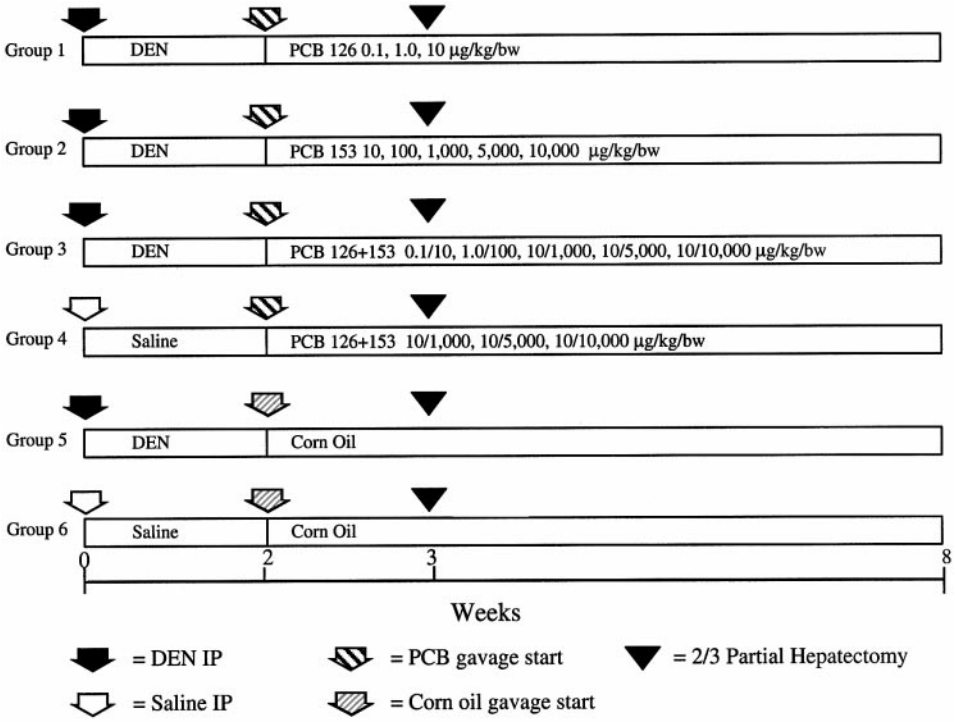

FIG. 1. Experimental design for the initiation/promotion study with PCBs. Diethylnitrosamine (DEN), the initiation agent, was given via ip injection (200 mg/kg) at Day 0. PCB treatment 3 times a week via oral gavage began at Week 2 and continued until completion of the study at Week 8.

the carcinogenicity of PCBs in humans has not been established, the carcinogenic effects of individual PCBs and mixtures of PCBs in laboratory animals have been amply demonstrated (Mayes *et al.*, 1998).

PCBs in the environment are mixtures of congeners with differing physical and biological characteristics and their impact on biological systems may be due to interactions between different congeners. Despite this, there are relatively few relevant data on such interactive biological effects and risk estimation remains based on toxicologic data for individual PCB congeners (Safe, 1994). Understanding the interactions between PCB 126 and PCB 153 is important due to the potent hepatotoxicity of PCB 126 (Kimbrough, 1995) and the ubiquitous nature of PCB 153 (Cogliano, 1998; Hansen, 1998). This study was designed to evaluate and quantify the interactive hepatic tumor promoting effects of these two environmentally relevant PCBs.

MATERIALS AND METHODS

Chemicals. PCB 126 was purchased from Accustandard (New Haven, CT), PCB 153 was purchased from Ultra Scientific (North Kingstown, RI), and diethylnitrosamine (DEN) was purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Animals and care. Thirty-day-old, female F344 rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and allowed to acclimate to altitude (Fort Collins, CO; 5000 feet) for 4 weeks. Following acclimation, rats were randomized by weight and divided into treatment groups. All animals were housed (3 per cage) in polycarbonate cages with corncob bedding and stainless steel wire tops and were maintained at 25°C with 55% humidity with lighting maintained on a 12-h light/12-h dark cycle. Control and exposed animals were given food (Harland Tedkad NIH-07 diet; Madison, WI) and deionized water *ad libitum*. Rats were observed for clinical state twice daily, and food and water consumption and body weight were evaluated 3 times weekly. This study was conducted in accordance with the National Institutes of Health guidelines for the care of laboratory animals. Animals were housed in facilities fully accredited by the Association for Accreditation of Laboratory Animal Care.

Experimental design. To evaluate promotional potential we used a medium-term bioassay for promoters of hepatocarcinogenesis (Ito *et al.*, 1989). This assay employs placental glutathione-S-transferase positive (GST-P+) liver cell foci as markers of preneoplasia in the rat after treatment with the known initiator DEN followed by partial hepatectomy and exposure to a test chemical or chemical mixture. In contrast to some other known rat hepatic preneoplastic markers, such as glucose-6-phosphatase, adenosine triphosphatase, γ-glutamyltransferase and glucose-6-phosphatase dehydrogenase, GST-P appears to be more stable after withdrawal of carcinogens from the diet. Ease of visualization has also established GST-P as one of the best markers for detection of early liver lesions for analysis of hepatocarcinogenesis and in rapid bioassay methods for carcinogens. Although not all the liver foci detected may necessarily develop into tumors, good correlation (93% concordance) is obtained between the induction of GST-P positive foci and the incidence of hepatocellular carcinomas in parallel long-term assays. (Hasegawa and Ito, 1994). At the start of the study, rats received either a single ip injection of DEN (200 mg/kg) dissolved in 0.9% saline or an injection of saline only. Fourteen days after initial DEN/saline treatment, oral gavage administration of PCBs dissolved in 1 ml of corn oil vehicle or corn oil alone was begun. Several dose levels of the two PCBs in corn oil, either alone or in combination were used (Fig. 1). The low PCB doses were aimed at achieving levels roughly comparable to what has been found in human tissues. The high PCB doses (PCB 126 at 1.0 and 10 μg/kg and PCB 153 at 1000–10,000 μg/kg) were based on reported studies with PCB promotion of liver foci (Bager *et al.*, 1995; Hemming *et al.*, 1993, 1995). Groups 1 and 2 consisted of DEN-initiated single PCB treated animals. Group 3 contained DEN-initiated PCB combination treated animals. Group 4 contained non-DEN-initiated high dose individual and combined PCB treated animals. This group served to assay the potential of

TABLE 1
Body and Liver Weights in Diethylnitrosamine Initiated Rats Treated with PCBs 126 and 153 Alone and in Combination

Dose (μg/kg bw)	Body weight (g) ± SD	Liver weight (g) ± SD
DEN-corn oil		
0	165.66 ± 10.63	5.65 ± 0.47
PCB 126		
0.1	158.14 ± 9.31	5.36 ± 0.38
1.0	160.74 ± 10.48	5.94 ± 0.5
10	155.07 ± 17.71*	7.56 ± 1*
PCB 153		
10	157.25 ± 9.68	5.21 ± 0.56
100	156.92 ± 12.46**	5.63 ± 1.32
1000	158.9 ± 8.62	5.58 ± 0.35
5000	160.56 ± 7.93	7.02 ± 0.40*
10,000	167.04 ± 7.72	7.57 ± 0.86*
PCB mixture 126/153		
0.1/10	174.58 ± 7.76	6.27 ± 1.97
1/100	171.29 ± 8.49	6.01 ± 0.34
10/1000	163.89 ± 8.51	7.78 ± 0.57*
10/5000	153.38 ± 8.39*	10.14 ± 0.81*
10/10,000	150.04 ± 10.07*	10.57 ± 1043*

Note. Data expressed as mean ± SD. Weights at time of 8-week sacrifice.
* Significant difference ($p < 0.01$) from initiated control group.
** Significant difference ($p < 0.05$) from initiated control group.

high dose PCBs alone and in combination to induce GST-P foci without prior DEN exposure. Groups 5 and 6 contained the DEN control and vehicle control animals respectively. On Day 21 after initial DEN/saline treatment, a 2/3 partial hepatectomy was performed on all animals. Gavages were administered 3 times weekly through the remainder of the 8-week study. On Day 56 after initial treatment, rats were sacrificed by exsanguination via the abdominal aorta while under isoflorane anesthesia.

Immunohistochemical staining and liver foci analysis. A total of 4 liver sections were taken for examination from each animal, one each from the right posterior lobe and caudate lobe and two from the right anterior lobe. Sections were fixed in 10% neutral buffered formalin for immunohistochemical examination for GST-P+ foci. Liver sections were embedded in paraffin and sectioned at 5 μm. For antigen retrieval, liver sections were microwaved on high power until boiling in a 1:10 dilution citrate buffer (Biogenex Labs, San Ramon, CA). After boiling, sections were microwaved at a reduced power for 10 min. A standard avidin biotin (ABC) protocol was followed using an immunoperoxidase kit (Vector Laboratories, Burlingame, CA). GST-P primary antibody (Binding Site, San Diego, CA) incubations were performed at 37°C for 1 h. A final incubation with 3-amino-9-ethyl carbazole (AEC; Biomeda, Foster City, CA) was then carried out. Slides were counterstained with hematoxylin for histologic evaluation.

Number and area of GST-P+ foci greater than 0.2 mm diameter per liver sections examined were quantified. GST-P+ foci were measured using a Leitz light microscope coupled with the Bioquant® IV computerized histomorphometry program (B and M Biometrics, Nashville, TN). These measurements consisted of manually outlining the GST-P+ foci on the liver sections and allowing software to compute the enclosed area. Reference areas of liver sections were measured on a separate system using a Dage CCD72 MTI camera (Dage Corporation, Michigan City, IN) connected to a Bioquant® for Windows system (Version 2.6). In this system, images of the liver sections were projected on a computer terminal and automatically outlined and measured by the computer software. Results for the treatment and control groups were expressed as foci area in mm^2 of GST-P+ foci more than 0.2 mm in diameter per area of liver sections examined in cm^2. The use of foci > 0.2 mm has been shown to provide the best correlation with the development of hepatocellular carcinoma (Tatematsu *et al.,* 1988).

Analysis of PCBs in the liver and adipose tissue. Samples of liver and fat from animals were analyzed for their PCB content. Tissues were extracted by a modification of published procedures (de Faubert Maunder *et al.,* 1964; Mills *et al.,* 1963). PCB isomer determinations were by gas chromatography and ^{63}Ni pulsed electron-capture detector operated at 32°C. Analysis was performed with dual column confirmation using a 0.25 mm × 60 m DB-5 capillary column and a 0.32 mm × 30 m DB-608 capillary column. Each set of 10 samples included one reagent blank and a control spike of calibration standard. Evaluation of data was based on residues found in the reagent blank, recovery of a matrix spike of all analytes, and recovery of a PCB (BZ#183) spike from all samples.

Statistical analysis. Numbers and areas of GST-P foci greater than 0.2 mm in diameter were analyzed using two-way ANOVA for unequal group size, with PCB 126 dose and PCB 153 dose as the 2 main effects including their interaction. This analysis was followed by post hoc comparisons of the PCB treatment group means compared to DEN-corn oil control via Dunnett's multiple comparison procedure. PCB tissue concentrations and changes in liver and body weights were analyzed by one-way ANOVA using Tukey's pairwise comparisons procedure where DEN-initiated, PCB-treated animals were compared to DEN-corn oil controls. All statistical tests were performed using the Minitab statistical software package, release 10.5 (Minitab, State College, PA).

RESULTS

Body and Liver Weight

No adverse clinical effects were observed in any of the treatment groups during the experimental period. Table 1 summarizes final body weight and absolute liver weights at the end of the 8-week study. Body weights were significantly decreased ($p < 0.01$) in the PCB 126-treated animals (10 μg/kg) when compared to DEN/corn oil-treated controls. Body weights of PCB 153 treated rats were decreased, ($p < 0.05$) only in the group dosed at 100 μg/kg. Body weights were decreased in the two highest PCB 126/PCB 153 mixture-treated groups, (10/5000 and 10/10,000 μg/kg) when compared to DEN/corn oil-treated controls. Liver weights were increased

TABLE 2
Liver Concentrations of PCB 126 after Treatment with PCB 126 Alone and in Combination with PCB 153 in Diethylnitrosamine-Treated Rats

	PCB 126 (ng/g liver)		
Dose (μg/kg)	0.1	1.0	10
PCB 153			
0	7.2 ± 8.3	110.9 ± 34.1	909 ± 141.3
10	15.2 ± 2.0	—	—
100	—	101.6 ± 14.5	—
1000	—	—	1030 ± 131.5
5000	—	—	764.8 ± 76.7
10,000	—	—	970 ± 403

Note. PCB 126 liver concentrations after combined PCB 126/153 exposure were not significantly different ($p > 0.05$) at any dose compared with PCB 126 exposure alone.

TABLE 3
Liver Concentrations of PCB 153 after Treatment with PCB 153 Alone and in Combination with PCB 126 in Diethylnitrosamine-Treated Rats

	PCB 153 (ng/g liver)				
Dose (μg/kg)	10	100	1000	5000	10,000
PCB 126					
0	38.7 ± 7.0	250.6 ± 87.1	3843 ± 451	14,483 ± 3243	29,850 ± 6148
0.1	43.3 ± 7.6	—	—	—	—
1.0	—	326 ± 82.5	—	—	—
10	—	—	5913 ± 972*	28,200 ± 6148*	75,900 ± 19,107*

* Significantly increased ($p < 0.01$) compared with PCB 153 exposure alone.

($p < 0.01$) in the highest dose PCB 126 group (10 μg/kg), the two highest dose PCB 153 groups (5000 and 10,000 μg/kg) and the 3 highest PCB 126/PCB 153 mixture groups (10/1000, 10/5000, and 10/10,000 μg/kg). Relative liver weights (liver weight as % of body weight) demonstrated similar changes as absolute liver weights (data not shown).

Liver and Adipose Tissue Concentrations of PCBs

Tables 2–5 summarize liver and adipose tissue PCB concentrations at the time of sacrifice.

Liver concentrations increased in a dose dependent manner in rats treated with PCB 126 alone (Table 2). After combined PCB 126/153 exposure, PCB 126 liver concentrations were not significantly different at any dose compared with PCB 126 exposure alone (Table 2). Similarly, liver concentrations also increased in a dose dependent manner in rats treated with PCB 153 alone (Table 3). However, after combined PCB 126/153 exposure (Table 3, bottom row) PCB 153 levels were significantly increased ($p < 0.01$) at the 3 highest mixture doses compared with PCB 153 exposure alone.

Adipose tissue levels increased in a dose dependent manner in rats treated with PCB 126 or PCB 153 alone (Tables 4 and 5). PCB 126 fat concentration was significantly increased ($p < 0.05$) compared with PCB 126 exposure alone only after one mixture exposure (PCB 126 1.0 μg/kg + PCB 153 100 μg/kg; Table 4). PCB 153 fat concentrations after combined PCB 126/153 exposure were not significantly different at any dose compared with PCB 153 exposure alone (Table 5).

TABLE 4
Adipose Tissue Concentrations of PCB 126 after Treatment with PCB 126 Alone and in Combination with PCB 153 in Diethylnitrosamine-Treated Rats

	PCB 126 (ng/g fat)		
Dose (μg/kg)	0.1	1.0	10
PCB 153			
0	2.2 ± 4.4	13.7 ± 0.6	91.4 ± 26.6
10	4.0 ± 3.6	—	—
100	—	15.5 ± 1.5*	—
1000	—	—	53.1 ± 11.3
5000	—	—	64.1 ± 12.8
10,000	—	—	64.4 ± 4.1

* Significantly increased ($p < 0.05$) compared with PCB 126 exposure alone.

Quantitative Morphometric Evaluation of GST-P Positive Foci

Treatment of non-DEN initiated rats with PCBs alone or in combination did not result in formation of GST+ foci (Group 4, data not shown), thus indicating that the PCBs alone did not have initiating potential in our study. Treatment with PCB 126 or PCB 153 alone resulted in a significant ($p < 0.01$) dose dependent increase in GST-P+ foci area and number compared with DEN controls (Figs. 2 and 3) at the highest doses. Treatment with the mixture of PCB 126 and 153 resulted in significantly increased foci area and number ($p < 0.01$) in the 3 highest dose groups (Fig. 4). When the two PCBs were combined, PCB 153 had an antagonistic effect on PCB 126 GST-P formation. The interaction between the two PCBs after subtraction of the DEN control responses from the PCB-induced responses is shown in Figure 5. This response was less than additive and analysis (two-way ANOVA using a general linear model) showed this negative interaction to be significant ($p < 0.001$). PCB mixture responses were less than the predicted additive values of the individual PCBs for all dose groups.

DISCUSSION

In carcinogen-treated rats, single hepatocytes that express the placental form of glutathione-S-transferase (GST-P) represent putative initiated cells that have not yet been promoted (Dragan *et al.*, 1995; Moore *et al.*, 1987). Altered hepatocellular foci that consist of hyperplastic enzyme-altered cells that stain for GST-P are considered to be preneoplastic in nature (Pitot, 1990) and are the most likely to progress to form true neoplasms in the rodent (Hasegawa and Ito, 1994; Ogiso *et al.*,

TABLE 5
Adipose Tissue Concentrations of PCB 153 after Treatment with PCB 153 Alone and in Combination with PCB 126 in Diethylnitrosamine-Treated Rats

	PCB 153 (ng/g fat)				
Dose (μg/kg)	10	100	1000	5000	10,000
PCB 126					
0	872 ± 107*	7245 ± 1524	72,050 ± 11,468	325,167 ± 98,146	651,667 ± 241,137
0.1	716 ± 128	—	—	—	—
1.0	—	6358 ± 416	—	—	—
10	—	—	54,860 ± 10,992	407,500 ± 180,495	648,333 ± 181,112

Note. PCB 153 fat concentrations after combined PCB 126/153 exposure were not significantly different ($p > 0.05$) at any dose compared with PCB 153 exposure alone.

1990). Although preneoplastic foci are reversible and most regress, some foci continue to progress to adenomas and carcinomas (Grisham, 1997). The formation of foci can be accelerated using protocols that combine treatment with an initiator, followed by cell proliferation stimulated by partial hepatectomy, and other chemicals as agents of promotion (Dragan *et al.*, 1995; Ito *et al.*, 1989; Pitot and Dragan, 1995). This study evaluated hepatic tumor promoting activity induced by the AhR agonist PCB 126 and CAR agonist PCB 153. Treatment of non-DEN initiated rats with PCBs alone or in combination did not result in formation of GST+ foci (Group 4, data not shown), thus indicating that the PCBs alone did not have initiating potential in our study. The two PCB congeners alone enhanced GST-P+ altered hepatic foci at the highest doses administered, while PCB 153 had an antagonistic effect on PCB 126 GST-P formation when the two PCBs were combined.

Mixtures of AhR agonist and CAR agonist PCB congeners have been reported to influence the occurrence of foci in different ways. A mixture of PCB 77 and PCB 47 was reported by Sargent *et al.* (1991) to give a seemingly synergistic effect in one dose combination. In a study by Bager *et al.* (1995), 3 different doses of PCB 126 in combination with one dose of PCB 153 also responded in a synergistic manner. Berberian *et al.* (1995), however, did not observe any synergistic effects on foci following PCB 77 and PCB 153 exposure, but did report the number and volume of altered hepatic foci were increased by both congeners individually. Our findings that both AhR agonist and CAR agonist PCB congeners can act as promoters of carcinogenesis individually are consistent with the findings of Berberian *et al.* (1995) and other investigators (Buchmann *et al.*, 1986, 1991; Cogliano, 1998; Haag-Grönlund *et al.*, 1997, 1998; Safe, 1994; Silberhorn *et al.*, 1990). Our finding of antagonistic foci formation differed from reported studies of synergism between these two PCB congeners and other mixtures studies with more planar and less planar congeners (Bager *et al.*, 1995; Sargent *et al.*, 1991). This finding was surprising because our studies were based on similar reported

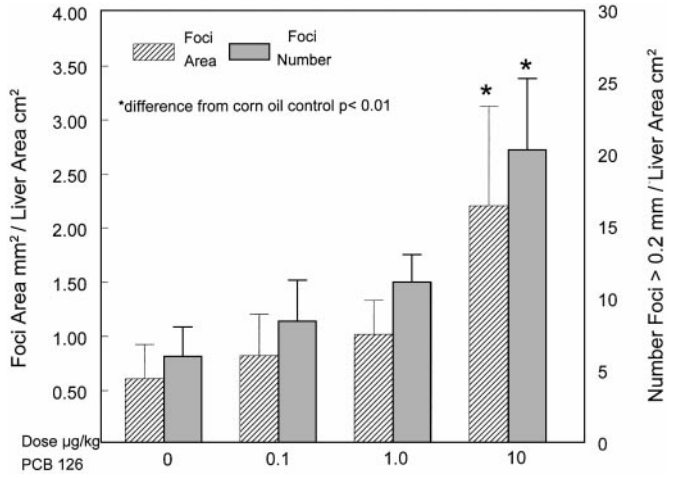

FIG. 2. Area and number of GST-P positive foci (>0.2 mm diameter) in female F344 rats subjected to an initiation/promotion protocol using diethylnitrosamine (DEN) as an initiator and PCB 126 as a promoter agent.

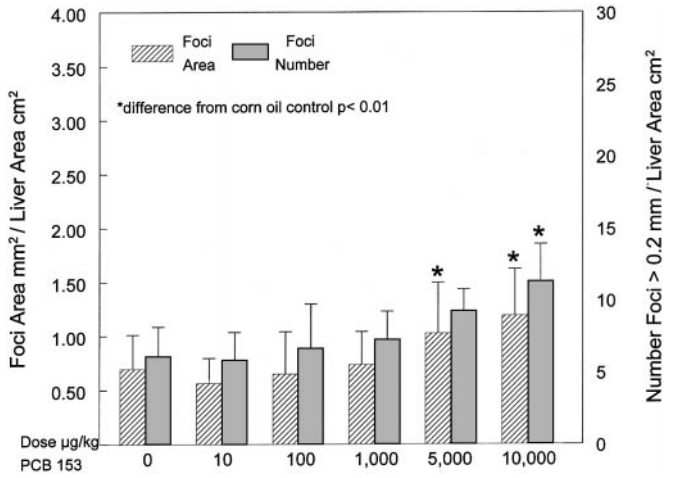

FIG. 3. Area and number of GST-P positive foci (>0.2 mm diameter) in female F344 rats subjected to an initiation/promotion protocol using diethylnitrosamine (DEN) as an initiator and PCB 153 as a promoter agent.

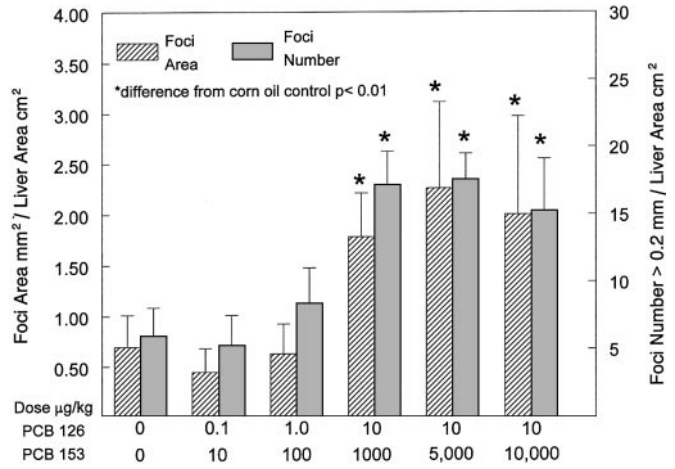

FIG. 4. Area and number of GST-P positive foci (>0.2 mm diameter) in female F344 rats subjected to an initiation/promotion protocol using diethylnitrosamine (DEN) as an initiator and a mixture of PCB 126 and PCB 153 as a promoter.

dosing schemes (Bager *et al.,* 1995). A more recent publication, however, is in accordance with our findings of antagonism (Haag-Grönlund *et al.,* 1998). Haag-Grönlund *et al.* (1998) reported a combination of PCB 126 and PCB 153 resulted in a weak antagonistic effect for volume fraction of altered hepatic foci. Interestingly, van der Plas *et al.* (1999) report that addition of PCB 153 to a mixture of AhR agonist polyhalogenated aromatic hydrocarbons, including TCDD and PCB 126, resulted in a higher mean foci volume and volume fraction when compared to the mixture without PCB 153, thus suggesting additivity.

While there has been extensive research on carcinogenicity of dioxins and dioxin-like AhR agonist PCBs, the actions of the CAR agonist PCB congeners are less well studied, as are potential interactions between the 2. Although it is accepted that dioxin-like PCBs mediate their toxic effects via the Ah receptor, little is known about the mechanisms of action of CAR agonist congeners. The varied results obtained from mixtures of AhR agonist and CAR agonist PCBs challenge the hypothesis that the AhR route is the only mechanism involved in tumor promotion by PCB and suggest that other mechanisms may play an important role in the interactive tumor promoting effects of PCBs.

It has been stressed that nonadditive interactions between AhR agonist PCBs or TCDD with CAR agonist PCBs appear to be affected by toxicokinetic interactions (van den Berg *et al.,* 1994) and that knowledge of actual tissue retention is critical both for understanding mechanisms and for providing realistic risk assessments (Giesy and Kannan, 1998). TCDD binding in liver is related to induction of a specific hepatic binding protein, CYP1A2, and by binding the AhR (van Birgelen *et al.,* 1996). Selective binding of the AhR by PCB 126 also occurs (Chu *et al.,* 1996). High doses of PCB 153 are reported to alter the distribution of TCDD, increasing the concentration in liver and decreasing the concentration in adipose tissues via induction of hepatic CYP1A2, which subsequently binds TCDD (De Jongh *et al.,* 1995). PCB 153 concentration can be increased in liver by a high dose of TCDD (van Birgelen *et al.,* 1996). PCB 153 is lipophilic and, since TCDD appears to increase PCB 153 levels in the liver, it has been suggested that this is related to increased fat in liver cells due to toxicity of the TCDD (van Birgelen *et al.,* 1996).

Our data indicate an increase in PCB 153 concentrations in liver when administered in combination with PCB 126 compared with PCB 153 alone. Increased levels of PCB 153 in the liver would be expected to increase promotional effects based on the effect of the chemical alone, rather than to decrease them, as we have seen. Thus, distribution changes alone could not account for the antagonistic promotional effects. While the mechanism of this distribution change is not clear, the rats in the highest dose groups (PCB 126 at 10 μg/kg and PCB 153 at 5000 and 10,000 μg/kg) did have significant weight loss compared to controls. Loss of adipose tissue could have led to redistribution of the PCB 153 from the fat to the liver. As previously mentioned, one study suggested that TCDD toxicity caused hepatic lipidosis and this increased PCB 153 levels in liver because of its lipophilicity (van Birgelen *et al.,* 1996). This is unlikely in our study, since we saw no histologic evidence of hepatic toxicity or visible fat increase after exposure to individual congeners or mixtures. PCB 126 fat concentration after combined PCB 126/153 exposure increased only at one dose combination (PCB 126 1.0 μg/kg + PCB 153 100 μg/kg) and this was a minimal change. The specific congeners in the mixture, the dose levels of the individual congeners, route of administration, duration of exposure, and species and strain of animal no doubt affect such toxicokinetic interactions. Regardless, our results suggest toxicokinetic interactions alone cannot explain the antagonistic promotional response seen with the PCB mixture.

Understanding the mechanism of interaction between PCB

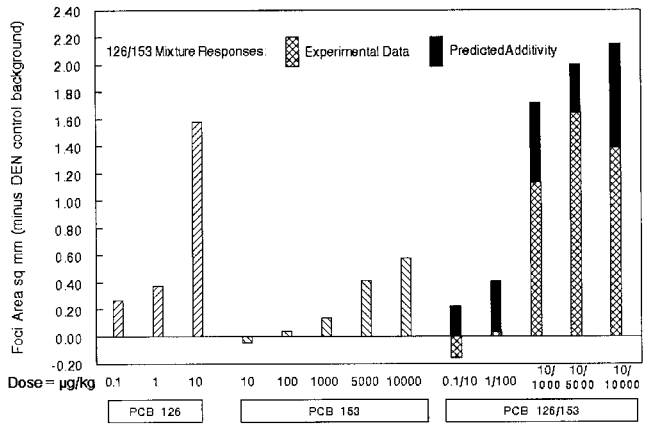

FIG. 5. Total effect in each treatment group (area of GST-P positive foci with background DEN response subtracted) after treatment with PCB 126, 153, and PCB 126 and 153 combined.

126 and PCB 153 is important because they are among the most environmentally important congeners (Kimbrough, 1995; McFarland and Clarke, 1989) and are found in significant quantities in human tissues (Cogliano, 1998; Hansen, 1998). Currently risk estimation is based on toxicologic data on individual PCBs, mainly the AhR agonist congeners (Giesy and Kannan 1998; Safe, 1994; van den Berg *et al.*, 1998; Whysner and Williams, 1996). The toxic equivalency factor (TEF) concept is used to facilitate the risk assessment of PCBs and related substances, the potency of the most toxic dioxin, TCDD, is used as a standard and set to 1, and other dioxin-like chemicals are assigned TEFs that are fractions thereof. The total toxic equivalency (TEQ) of a mixture is the sum of the individual potencies. Several assumptions are made when utilizing the TEF scheme. First, all interactions are presumed to be additive in nature even though nonadditive PCB interactions are reported. TEFs are assigned based on a chemical's affinity to bind to the AhR, which CAR agonist PCB congers are unable to do. Therefore, the current system does not include the potential impact of CAR agonist PCB congeners even though they are known promoters. It has been stressed that without consideration of the toxicity of the CAR agonist PCBs, current risk assessment for the PCBs is incomplete (Fischer *et al.*, 1998; Hansen, 1998; van den Berg *et al.*, 1998). With respect to this, our lowest dose of PCB 153 (10 μg/kg body weight) resulted in adipose tissue levels of 872 $\pm$ 107 ng/g, only 2–3 times those reported in human tissues (Hansen, 1998; Stellman *et al.*, 1998). Our low dose of PCB 126 (0.1 μg/kg body weight) resulted in tissue levels that were at the lower limit of detection by our analysis system, which is highly sensitive (capable of detecting 8 ng/g and above), again emphasizing the environmental relevance of these studies. Our data demonstrate antagonistic hepatic tumor promoting activity in a mixture of AhR agonist and CAR agonist PCBs, which results in a less than additive GST-P foci formation. The less than additive response was present in all 5 PCB 126/PCB 153 combinations, including low doses of PCB 153 (10 and 100 μg/kg) that alone did not show any evidence of promotional activity (Fig. 5).

In summary, results from this study confirm that individual treatment with either the CAR agonist PCB 153 or AhR agonist PCB 126 lead to the formation of GST-P+ altered enzymatic hepatic foci and that both congeners can act as promoters of hepatocarcinogenesis. Additionally, treatment with a mixture of PCB 126 and 153 resulted in less than additive promotional interactions. These results suggest that risk estimation for mixtures containing multiple PCBs cannot be based on a model of simple additivity as has been proposed.

ACKNOWLEDGMENTS

We thank the many individuals at the Center for Environmental Toxicology and Technology and the Painter Laboratory Animal Facility at Colorado State University for their assistance in carrying out these studies. This research was supported by grants from the National Institute of Environmental Health Sciences Superfund Basic Research Program Project, P42-ES05949, and National Institute of Environmental Health Sciences Mentored Clinical Scientist Development Award, K08ES00314.

REFERENCES

Bager, Y., Hemming, H., Flodström, S., Ahlborg, U. G., and Wärngård, L. (1995). Interaction of 3,4,5,3′,4′-pentachlorobiphenyl and 2,4,5,2′,4′,5′-hexachlorobiphenyl in promotion of altered hepatic foci in rats. *Pharmacol. Toxicol.* **77,** 149–154.

Berberian, I., Chen, L. C., Robinson, F. R., Glauert, H. P., Chow, C. K., and Robertson, L. W. (1995). Effect of dietary retinyl palmitate on the promotion of altered hepatic foci by 3,3′,4,4′-tetrachlorobiphenyl and 2,2′,4,4′,5,5′-hexachlorobiphenyl in rats initiated with diethylnitrosamine. *Carcinogenesis* **16,** 393-398.

Buchmann, A., Ziegler, S., Wolf, A., Robertson, L. W., Durham, S. K., and Schwarz, M. (1991). Effects of polychlorinated biphenyls in rat liver: Correlation between primary subcellular effects and promoting activity. *Toxicol. Appl. Pharmacol.* **111,** 454–468.

Buchmann, A., Kunz, W., Wolf, C. R., Oesch, F., and Robertson, L. W. (1986). Polychlorinated biphenyls, classified as either phenobarbital- or 3-methylcholanthrene-type inducers of cytochrome P-450, are both hepatic tumor promoters in diethylnitrosamine-initiated rats. *Cancer Lett.* **32,** 243–253.

Chu, I., Villeneuve, D. C., Yagminas, A., Lecavalier, P., Poon, R., Feeley, M., Kennedy, S. W., Seegal, R. F., Håkansson, H., Ahlborg, U. G., Valli, V. E., and Bergman, Å. (1996). Toxicity of 2,2′,4,4′,5,5′-hexachlorobiphenyl in rats: Effects following 90-day oral exposure. *J. Appl. Toxicol.* **16**, 121–128.

Cogliano, V. J. (1998). Assessing the cancer risk from environmental PCBs. *Environ. Health Perspect.* **106,** 317–323.

de Faubert Maunder, M. J., Egan, H., Godly, E. W., Hammond, E. W., Roburn, J., and Thompson, J. (1964). Clean-up of animal fats and dairy products for the analysis of chlorinated pesticide residues. *Analyst* **89,** 168–170.

De Jongh, J., DeVito, M., Nieboer, R., Birnbaum, L., and Van den Berg, M. (1995). Induction of cytochrome P450 enzymes after toxicokinetic interactions between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 2,2′,4,4′,5,5′-hexachlorobiphenyl in the liver of the mouse. *Fundam. Appl. Toxicol.* **25,** 264–270.

Dragan, Y., Teeguarden, J., Campbell, H., Hsia, S., and Pitot, H. (1995). The quantitation of altered hepatic foci during multistage hepatocarcinogenesis in the rat: Transforming growth factor a expression as a marker for the stage of progression. *Cancer Lett.* **93,** 73–83.

Fischer, L. J., Seegal, R. F., Ganey, P. E., Pessah, I. N., and Kodavanti, P. R. (1998). Symposium overview: Toxicity of non-coplanar PCBs. *Toxicol. Sci.* **41,** 49–61.

Giesy, J. P., and Kannan, K. (1998). Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): Implications for risk assessment. *Crit. Rev. Toxicol.* **28,** 511–569.

Grisham, J. W. (1997). Interspecies comparison of liver carcinogenesis: Implications for cancer risk assessment. *Carcinogenesis* **18,** 59–81.

Haag-Grönlund, M., Johansson, N., Fransson-Steen, R., Håkansson, H., Scheu, G., and Wärngård, L. (1998). Interactive effects of three structurally different polychlorinated biphenyls in a rat liver tumor promotion bioassay. *Toxicol. Appl. Pharmacol.* **152,** 153–165.

Haag-Grönlund, M., Kato, Y., Fransson-Steen, R., Scheu, G., and Wärngård, L. (1997). Promotion of enzyme altered foci in female rat by 2,3,3′,4,4′,5-hexachlorobiphenyl. *Toxicol. Appl. Pharmacol.* **147,** 46–55.

Hansen, L. G. (1998). Stepping backward to improve assessment of PCB congener toxicities. *Environ. Health Perspect.* **106**(Suppl. 1), 171–189.

Hasegawa, R., and Ito, N. (1994). Hepatocarcinogenesis in the rat. In *Carcinogenesis* (M. P. Waalkes and J. M. Ward, Eds.), pp. 39–64. Raven Press, New York.

Hemming, H., Bager, Y., Flodström, S., Nordgren, I., Kronevi, T., Ahlborg,

U. G., and Wärngård, L. (1995). Liver tumour promoting activity of 3,4,5,3′,4′-pentachlorobiphenyl and its interaction with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Eur. J. Pharmacol.* **292,** 241–249.

Hemming, H., Flodström, S., Wärngård, L., Bergman, Å., Kronevi, T., Nordgren, I., and Ahlborg, U. G. (1993). Relative tumor promoting activity of three polychlorinated biphenyls in rat liver. *Eur. J. Pharmacol.* **248,** 163–174.

Ito, N., Tatematsu, M., Hasegawa, R., and Tsuda, H. (1989). Medium-term bioassay system for detection of carcinogens and modifiers of hepatocarcinogenesis utilizing the GST-P positive liver cell focus as an endpoint marker. *Toxicol. Pathol.* **17,** 630–641.

Kimbrough, R. D. (1995). Polychlorinated biphenyls (PCBs) and human health: An update. *Crit. Rev. Toxicol.* **25,** 133–163.

Mayes, B. A., McConnell, E. E., Neal, B. H., Brunner, M. J., Hamilton, S. B., Sullivan, T. M., Peters, A. C., Ryan, M. J., Toft, J. D., Singer, A. W., Brown, J. F., Jr., Menton, R. G., and Moore, J. A. (1998). Comparative carcinogenicity in Sprague-Dawley rats of the polychlorinated biphenyl mixtures Aroclors 1016, 1242, 1254, and 1260. *Toxicol. Sci.* **41,** 62–76.

McFarland, V. A., and Clarke, J. U. (1989). Environmental ocurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: Considerations for a congener-specific analysis. *Environ. Health Perspect.* **81,** 225-239.

Mills, P. A., Onley, J. H., and Gaither, R. A. (1963). Rapid method for chlorinated pesticide residues in nonfatty foods. *J. Assoc. Official Analyt. Chem.* **46,** 186–191.

Moore, M. A., Nakagawa, K., Satoh, K., Ishikawa, T., and Sato, K. (1987). Single GST-P positive liver cells—putative initiated hepatocytes. *Carcinogenesis* **8,** 483–486.

Muangmoonchai, R., Smirlis, D., Wong, S. C., Edwards, M., Phillips, I. R., and Shephard, E. A. (2001). Xenobiotic induction of cytochrome P450 2B1 (CYP2B1) is mediated by the orphan nuclear receptor constitutive androstane receptor (CAR) and requires steroid co-activator 1 (SRC-1) and the transcription factor Sp1. *Biochem. J.* **355,** 71–78.

Ogiso, T., Tatematsu, M., Tamano, S., Hasegawa, R., and Ito, N. (1990). Correlation between medium-term liver bioassay system data and results of long-term testing in rats. *Carcinogenesis* **11,** 561–566.

Pitot, H. C. (1990). Altered hepatic foci: Their role in murine hepatocarcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **30,** 465–500.

Pitot, H. C., and Dragan, Y. P. (1993). Stage of tumor progression, progressor agents, and human risk. *Proc. Soc. Exp. Biol. Med.* **202,** 37–43.

Safe, S. (1984). Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): Biochemistry, toxicology, and mechanism of action. *Crit. Rev. Toxicol.* **12,** 319–395.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PC-DDs), dibenzofurans (PCDFs) and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit. Rev. Toxicol.* **21,** 51–88.

Safe, S. H. (1994). Polychlorinated biphenyls (PCBs): Environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit. Rev. Toxicol.* **24,** 87–149.

Safe, S., Safe, L., and Mullin, M. (1985). Polychlorinated biphenyls (PCBs) - Congener-specific analysis of a commercial mixture and a human milk extract. *J. Agric. Food Chem.* **33,** 24.

Sargent, L., Dragan, Y. P., Erickson, C., Laufer, C. J., and Pitot, H. C. (1991). Study of the separate and combined effects of the non-planar 2,5,2′,5′- and the planar 3,4,3′,4′-tetrachlorobiphenyl in liver and lymphocytes *in vivo*. *Carcinogenesis* **12,** 793–800.

Silberhorn, E. M., Glauert, H. P., and Robertson, L. W. (1990). Carcinogenicity of polyhalogenated biphenyls: PCBs and PBBs. *Crit. Rev. Toxicol.* **20,** 439-496.

Stellman, S. D., Djordjevic, M. V., Muscat, J. E., Gong, L., Bernstein, D., Citron, M. L., White, A., Kemeny, M., Busch, E., and Nafziger, A. N. (1998). Relative abundance of organochlorine pesticides and polychlorinated biphenyls in adipose tissue and serum of women in Long Island, New York. *Cancer Epidemiol. Biomarkers Prev.* **7,** 489–496.

Tatematsu, M., Mera, Y., Inoue, T., Satoh, K., Sato, K., and Ito, N. (1988). Stable phenotypic expression of glutathione S-transferase placental type and unstable phenotypic expression of gamma-glutamyltransferase in rat liver preneoplastic and neoplastic lesions. *Carcinogenesis* **9**, 215–220.

van Birgelen, A. P. J. M., Ross, D. G., DeVito, M. J., and Birnbaum, L. S. (1996). Interactive effects between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 2,2′,4,4′,5,5′-hexachlorobiphenyl in female B6C3F1 mice: Tissue distribution and tissue specific enzyme induction. *Fund. Appl. Toxicol.* **34,** 118–131.

Van den Berg, M., Birnbaum, L., Bosveld, A. T. C., Brunström, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., ven Leeuwen, F. X. R., Liem, A. K. D., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect.* **106,** 775–792.

Van den Berg, M., De Jongh, J., Poiger, H., and Olson, J. R. (1994). The toxicokinetics and metabolism of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) and their relevance for toxicity. *Crit. Rev. Toxicol.* **24,** 1–74.

van der Plas, S. A., Haag-Gronlund, M., Scheu, G., Warngard, L., van den Berg, M., Wester, P., Koeman, J. H., and Brouwer, A. (1999). Induction of altered hepatic foci by a mixture of dioxin-like compounds with and without 2,2′,4,4′,5,5′-hexachlorobiphenyl in female Sprague-Dawley rats. *Toxicol. Appl. Pharmacol.* **156,** 30–39.

Whysner, J., and Williams, G. M. (1996). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin mechanistic data and risk assessment: Gene regulation, cytotoxicity, enhanced cell proliferation, and tumor promotion. *Pharmacol. Ther.* **71,** 193–223.

Yang, R. S. H., and Rauckman, E. J. (1987). Toxicological studies of chemical mixtures of environmental concern at the national toxicology program: Health effects of groundwater contaminants. *Toxicology* **47,** 15–34.