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RECONSTITUTED HUMAN BREAST MILK PCBs AS POTENT INDUCERS OF ARYL HYDROCARBON HYDROXYLASE

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SUMMARY

The major polychlorinated biphenyl (PCB) components identified in human breast milk have been synthesized and a reconstituted breast milk PCB mixture representing the average levels determined in the Osaka Prefecture in Japan has been prepared. The dose effecting the half-maximal (ED_{50}) induction of rat hepatic microsomal aryl hydrocarbon hydroxylase (AHH) for the reconstituted breast milk PCBs (ED_{50} ~12 $\mu mol \cdot kg^{-1}$) was approximately seven times less than the ED_{50} for the commercial PCB mixture, Kanechlor 500. The increased biological potency of the former mixture reflects the preferential bioconcentration of the toxic PCB congeners, 2,3,3',4,4'-penta-, 2,3',4,4',5-penta- and 2,3,3',4,4',5-hexachlorobiphenyl.

INTRODUCTION

Commercial polychlorinated biphenyls (PCBs) are complex mixtures of isomers and congeners which have been detected in almost every component of the global ecosystem including human adipose, blood and breast milk samples (1,2). The chemical properties and biodegradability of individual PCBs are dependent on their structures and it is not surprising that the gas-chromatographic (GC) profiles of PCB residues in humans (3), other environmental samples (4), and laboratory animals (5) exposed to commercial PCBs differ markedly from the commercial mixtures (7).

Recently the fourteen major PCB congeners present in Japanese breast milk have been identified and quantified (8) (for the Osaka Prefecture). The rela-

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Abbreviations: PCBs - polychlorinated biphenyls; PB - phenobarbitone; MC - methylcholanthrene; CO - carbon monoxide; EIC - ethylisocyanide; DMAP - 4-dimethylaminoantipyrine; AHH - aryl hydrocarbon hydroxylase; B[a]P - benzo[a]pyrene hydroxylase

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tively simple GC profile differed markedly from the more complex and widely used Japanese commercial PCB mixture, Kanechlor 500, which is one of the most probable sources of environmental contamination by PCBs in Japan. The composition of Kanechlor 500 is similar to that of the major North American commercial PCB pollutant, Aroclor 1254 (54% by weight of chlorine), and not surprisingly the GC profiles of Japanese and North American breast milk PCBs are similar.

For various classes of halogenated aromatic hydrocarbons, including PCBs, there exists a correlation between toxicity and the ability of an individual congener to induce the cytochrome P-448-dependent monooxygenase, aryl hydrocarbon hydroxylase (AHH) (9,10). For example, two toxic responses, thymic atrophy (10,11) and porphyria (12) have been shown to segregate with the *Ah* locus whose product, a high-affinity cytosolic receptor protein, mediates the induction of AHH (13,14). The levels of this cytosolic receptor are highest in the thymus (15) which is highly sensitive to the toxic effects of those halogenated aryl hydrocarbons which induce AHH, including certain PCBs (16-18); moreover, unlike many of the toxic responses, the induction of AHH is an easily measured, sensitive, dose-related and well-documented biochemical response to halogenated aryl hydrocarbons.

This study compares the activity of Kanechlor 500 and a reconstituted breast milk PCB mixture as inducers of AHH activity in rat hepatic microsomes.

MATERIALS AND METHODS

Syntheses

The PCB isomers and congeners were synthesized by the Cadogan (C) coupling of a chlorinated aniline (10-15 mmol) in excess chlorinated benzene (200-300 mmol) or by the Ullman (U) coupling of the appropriate iodochlorobenzenes as indicated in Table 1. The crude reaction products were purified by alumina/Florisil column chromatography and repeated thin-layer chromatography (TLC) with petroleum ether (b.p. 30-60°) as described (19). The identities of the PCB congeners were confirmed by their 60 or 220 MHz proton magnetic resonance (PMR) and mass spectra and their purities determined by gas chromatography as described (19). 2,2',3,4,4',5,5'-Heptachlorobiphenyl was the only congener with a purity <98.5%. However, this compound did not induce hepatic AHH activity when administered to rats at 150 $\mu mol \cdot kg^{-1}$ (i.e., ten times that present in the highest dose of the breast milk PCBs).

Chemicals and Biochemicals

The chlorinated anilines and chlorinated benzenes were purchased from the Aldrich Chemical Company; amyl nitrite and 4-chloroiodobenzene were purchased from Eastman Organics, 2,4-dichloroiodobenzene and 2,4,5-trichloroiodobenzene were synthesized from the corresponding anilines. 2,2',4,4',5,5'-Hexachlorobiphenyl was prepared from 2,2',5,5'-tetrachlorobenzidine as described (22). Cytochrome c (horse heart, type III), $NADP^+$, NADPH, D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase (Baker's yeast), 3-methylcholanthrene (MC), benzo[a]pyrene (B[a]P) and ethylisocyanide (EIC) were purchased from Sigma Chemical Company; 4-dimethylaminoantipyrine (DMAP) from Aldrich Chemical Company; carbon monoxide (CO) (research purity) from Matheson, and sodium phenobarbitone from the Ontario Veterinary College, Guelph. [^{3}H]-Benzo-[a]pyrene was obtained from New England Nuclear Company and purified by TLC developed in hexane.

Animal Treatment, Isolation of Microsomes and Assays

The commercial and reconstituted breast milk PCB preparations were dissolved in corn oil and injected ip into one-month-old male Wistar rats. Each PCB mixture was given to three rats on days 1 and 3 at doses of 3, 30, 75, 150 and 300 $\mu mol \cdot kg^{-1}$. In addition, the commercial mixture was also given on days 1 and 3 at a dosage of 500 $\mu mol \cdot kg^{-1}$. Animals were killed by cervical dislocation on day 6. The apparent molecular weights of Kanechlor 500 and the breast milk PCBs were taken to be 325 and 334, respectively. 3-Methylcholanthrene (MC)(100 $\mu mol \cdot kg^{-1}$) and phenobarbitone (PB)(400 $\mu mol \cdot kg^{-1}$) were administered either individually or together for 2 and 3 consecutive days, respectively, to groups of 10 rats, and the animals killed 24 hours after the last injection. Controls (n = 10) received corn oil (5 $ml \cdot kg^{-1}$). Microsomes were prepared from perfused livers by differential centrifugation and all assays were performed essentially as described (19, 20). AHH activity was measured by the radiometric method of DePierre et al. (23) as improved by Nesnow et al. (24). Statistical differences were calculated by the student's t test at the 1% level of significance ($P < 0.01$).

RESULTS AND DISCUSSION

As shown in Table 1, a human breast milk PCB mixture was reconstituted from thirteen synthetic PCB congeners based on their reported average concentrations in Japanese breast milk. With one exception, the purity of all the synthetic PCBs was >98.5% and the clean-up procedure incorporated a Florisil column chromatographic step to remove any possible chlorinated dibenzofuran by-products (19,20). The preparation of pure 2,2',3,4',5,5'-hexachlorobiphenyl was not successful, hence, this isomer was omitted from the reconstituted mixture. Based on structure-activity rules, this congener would be expected to be a weak PB-type inducer (9).

Table 2 compares the effects of various equimolar concentrations of the Japanese commercial PCB mixture, Kanechlor 500, and the reconstituted breast milk PCBs as inducers of hepatic microsomal drug-metabolizing enzymes in one-

Table 1

A SUMMARY OF THE RECONSTITUTED BREAST MILK PCB ISOMERS AND CONGENERS

PCB Structure	GC Purity (%)	PCB Concentration in Japanese Breast Milk (%)	PCB Concentration in Reconstituted Breast Milk Mixture (%)	Synthetic Methods
2,4,4'-Trichlorobiphenyl	98.5	8.4 ± 2.2	9.2	4-chloroiodobenzene/2,4-dichloroiodobenzene (U)
2,2',5,5'-Tetrachlorobiphenyl	99.0	2.0 ± 1.3	2.2	2,5-dichloroaniline/1,4-dichlorobenzene (C)
2,4,4',5-Tetrachlorobiphenyl	99.0	19.1 ± 2.6	20.1	4-chloroiodobenzene/2,4,5-trichloroiodobenzene (U)
2,2',4,5,5'-Pentachlorobiphenyl	98.5	2.8 ± 0.9	3.0	2,4,5-trichloroaniline/1,4-dichlorobenzene (C)
2,3',4,4',5-Pentachlorobiphenyl	99.0	11.8 ± 1.2	12.8	2,4,5-trichloroaniline/1,2-dichlorobenzene (C)
2,2',3,4',5,5'-Hexachlorobiphenyl	99.0	2.3 ± 0.3	-	-
2,3,3',4,4'-Pentachlorobiphenyl	99.0	3.5 ± 0.5	3.9	2,3,4-trichloroaniline/1,2-dichlorobenzene (C)
2,2',4,4',5,5'-Hexachlorobiphenyl	99.0	15.5 ± 0.4	16.3	2,2',5,5'-tetrachlorobenzidine diazotization
2,2',3,4,4',5'-Hexachlorobiphenyl	99.0	15.8 ± 0.7	16.9	2,4,5-trichloroaniline/1,2,3-trichlorobenzene (C)
2,2',3,4',5,5',6-Heptachlorobiphenyl	98.5	3.2 ± 0.6	3.3	2,4,5-trichloroaniline/1,2,4,5-tetrachlorobenzene (C)
2,2',3,4,4',5',6-Heptachlorobiphenyl	99.0	1.6 ± 0.3	1.7	2,4,5-trichloroaniline/1,2,3,5-tetrachlorobenzene (C)
2,3,3',4,4',5-Hexachlorobiphenyl	99.0	2.1 ± 0.7	2.3	3,4-dichloroaniline/1,2,3,4-tetrachlorobenzene (C)
2,2',3,4,4',5,5'-Heptachlorobiphenyl	90.0	5.1 ± 0.7	5.7	2,4,5-trichloroaniline/1,2,3,4-tetrachlorobenzene (C)
2,2',3,3',4,4',5-Heptachlorobiphenyl	98.5	2.3 ± 0.3	2.3	2,3,4-trichloroaniline/1,2,3,4-tetrachlorobenzene (C)
Total		95.5	99.9	

(U) Ullman coupling; (C) Cadogan coupling

Table 2

THE EFFECTS OF VARIOUS DOSES OF KANECHLOR 500 AND A RECONSTITUTED BREAST MILK PCB MIXTURE ON HEPATIC DRUG-METABOLIZING ENZYMES

Treatment	% Liver Wt. of Body Wt.	mg Protein g Liver^{-1}	Benzo[a]pyrene Hydroxylase nmol B[a]P metabolised mg protein^{-1} min^{-1}	DMAP N-Demethylase nmol HCHO formed mg protein^{-1}min^{-1}	NADPH-Cytochrome c Reductase nmol mg protein^{-1}min^{-1}	Cytochrome b_5 nmol mg protein^{-1}	Ethylmorpholide - Difference Spectrum: Cytochrome P-450 nmol mg protein^{-1} (Peak Maximum) (nm)	Peak 428 nm (A428-A500) Peak Maximum (nm)	Peak 455 nm (A455-A500) Peak Maximum (nm)	Peak Height Ratio (455/428 nm)
Corn Oil	3.98 ± 0.23	19.4 ± 2.1	164 ± 18	3.73 ± 0.20	67.4 ± 11.2	193 ± 22	0.672 ± 0.051 (450.0)	0.053 ± 0.008 (428.0)	0.026 ± 0.002 (455.0)	0.49 ± 0.03
Phenobarbitone (PB)	5.02 ± 0.67	27.3 ± 3.2	508 ± 44	9.20 ± 0.26	173 ± 18	286 ± 20	1.73 ± 0.095 (450.0)	0.104 ± 0.013 (428.0)	0.071 ± 0.004 (455.0)	0.69 ± 0.04
3-Methylcholanthrene (MC)	4.64 ± 0.51	20.5 ± 2.0	2480 ± 120	4.10 ± 0.30	64.7 ± 9.3	263 ± 18	1.36 ± 0.066 (448.0)	0.051 ± 0.009 (429.6)	0.093 ± 0.011 (453.0)	1.8 ± 0.08
PB + MC	6.15 ± 0.80	29.7 ± 3.6	2490 ± 160	9.01 ± 0.67	174 ± 24	314 ± 23	2.25 ± 0.102 (448.5)	0.134 ± 0.021 (429.0)	0.148 ± 0.014 (453.2)	1.1 ± 0.07
Kanechlor 500										
3	4.01 ± 0.27	20.7 ± 2.4	367 ± 31	3.98 ± 0.23	66.7 ± 8.3	248 ± 47	0.664 ± 0.032 (450.0)	0.067 ± 0.006 (428.0)	0.031 ± 0.001 (455.0)	0.47 ± 0.06
30	4.06 ± 0.39	25.8 ± 2.4	843 ± 63	5.41 ± 0.14	78.2 ± 10.1	303 ± 14	0.933 ± 0.067 (449.9)	0.087 ± 0.031 (428.0)	0.052 ± 0.052 (455.0)	0.61 ± 0.09
75	4.38 ± 0.32	24.5 ± 0.4	1160 ± 56	7.14 ± 0.39	104 ± 14	330 ± 26	1.20 ± 0.09 (449.1)	0.085 ± 0.040 (428.3)	0.065 ± 0 (453.9)	0.77 ± 0.04
150	5.06 ± 0.67	25.1 ± 2.1	1680 ± 150	9.22 ± 0.49	137 ± 9.6	393 ± 12	1.52 ± 0.19 (448.8)	0.105 ± 0.013 (428.7)	0.115 ± 0.039 (453.5)	1.1 ± 0.3
300	5.26 ± 0.13	25.8 ± 2.5	2110 ± 70	9.19 ± 0.49	156 ± 27	420 ± 26	2.03 ± 0.24 (448.5)	0.113 ± 0.010 (429.0)	0.130 ± 0.020 (453.2)	1.2 ± 0.2
500	6.02 ± 0.40	33.2 ± 2.9	2310 ± 90	9.28 ± 0.71	163 ± 22	410 ± 23	2.25 ± 0.18 (448.5)	0.121 ± 0.025 (429.0)	0.145 ± 0.018 (453.0)	1.2 ± 0.1
Breast Milk PCBs										
3	4.21 ± 0.43	25.2 ± 2.3	575 ± 105	4.71 ± 0.27	70.3 ± 8.5	287 ± 12	0.783 ± 0.058 (449.8)	0.073 ± 0.008 (428.0)	0.042 ± 0 (454.6)	0.58 ± 0.06
30	4.87 ± 0.17	26.9 ± 0.7	1670 ± 130	6.01 ± 0.09	89.1 ± 11.6	360 ± 42	1.33 ± 0.04 (448.7)	0.069 ± 0.016 (428.7)	0.074 ± 0.016 (453.9)	1.1 ± 0
75	5.32 ± 0.57	31.5 ± 1.4	2160 ± 60	7.96 ± 0.29	118 ± 21	396 ± 21	1.87 ± 0.21 (448.5)	0.102 ± 0.016 (429.4)	0.117 ± 0.021 (453.2)	1.1 ± 0.1
150	5.37 ± 0.43	26.7 ± 4.0	2410 ± 50	8.62 ± 0.74	153 ± 21	407 ± 6	2.05 ± 0.18 (448.4)	0.104 ± 0.019 (429.6)	0.123 ± 0.009 (453.0)	1.3 ± 0.2
300	6.22 ± 0.73	29.4 ± 3.9	2550 ± 120	9.26 ± 0.53	175 ± 17	393 ± 12	2.29 ± 0.03 (448.3)	0.104 ± 0.007 (429.6)	0.141 ± 0.006 (453.0)	1.4 ± 0.1

month-old male Wistar rats. These effects are further compared to those elicited by treatment with corn oil (control), PB, MC or PB+MC. Both PCB mixtures produced a pattern of liver enzyme induction which displayed the hallmarks of both PB induction (namely increased activities of DMAP N-demethylase and NADPH-cytochrome c reductase) and MC induction (namely increased AHH activity). The mixed-type inducing properties of both PCB mixtures was confirmed by their carbon monoxide- and ethylisocyanide-difference spectra; the qualitative and quantitative aspects of which were simulated by the coadministration of PB with MC.

With decreasing dose, a rapid diminution of the mixed-type activity was observed for Kanechlor 500 and, with the exception of a 2.2-fold increase in

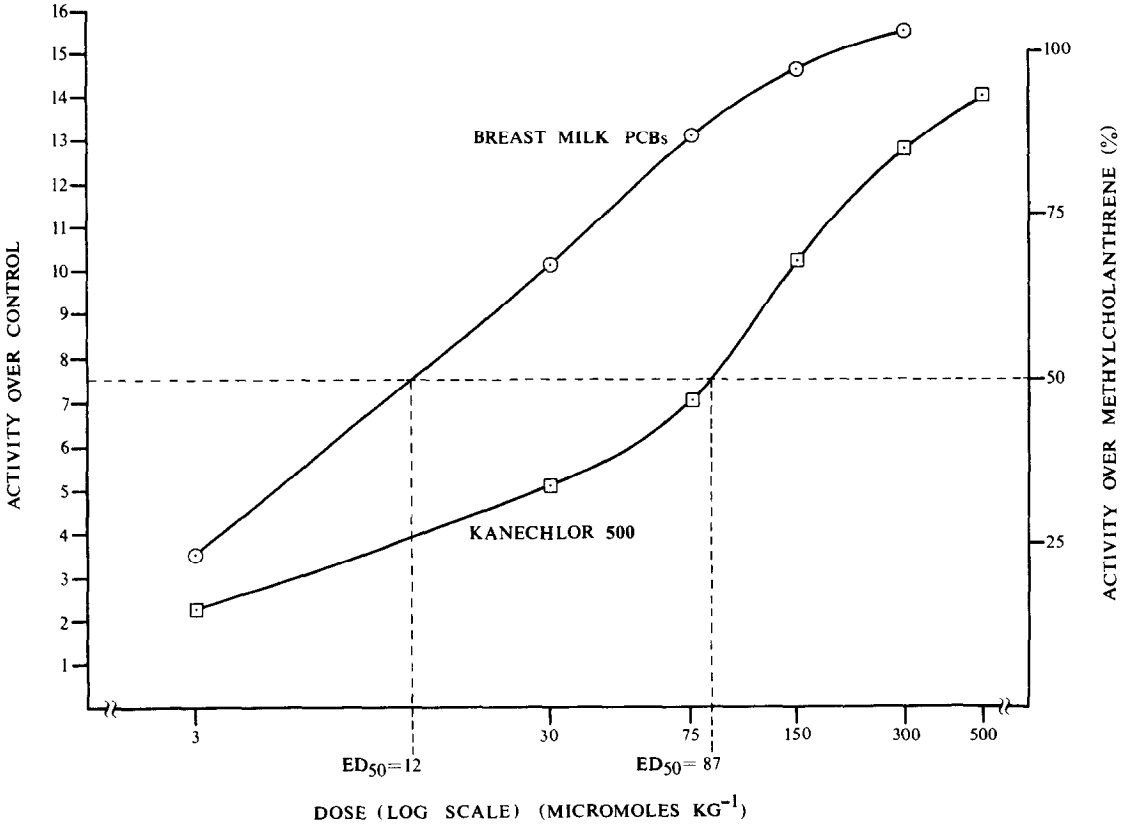

Figure 1. The variation of Aryl Hydrocarbon Hydroxylase (AHH) activity with the dose (log scale) of Kanechlor 500 (⊡) and a reconstituted human breast milk PCB mixture (⊙). The activity of AHH, measured by the rate of benzo-[a]pyrene hydroxylation (see Table 2), is expressed both as induced rate over control rate (left ordinate) and percentage of the MC-induced rate (right ordinate). Based on a value of 100% for the MC-induced rate, the dose effecting half-maximal induction of AHH (ED_{50}) is shown for each PCB preparation.

AHH activity, all parameters studied were not statistically different ($P < 0.01$) from control values at the lowest dose tested (3 $\mu mol \cdot kg^{-1}$). Although similar results were obtained for the reconstituted breast milk PCBs, a notably slower decline of mixed-type activity was observed with decreasing dose. Furthermore, in contrast to Kanechlor 500, most of the parameters studied were increased over control values even at the lowest dose tested. In particular, AHH activity was increased by 3.5-fold over controls.

Figure 1 shows the variation of AHH activity over a 167-fold and 100-fold dose range (plotted on a log scale) of Kanechlor 500 and of reconstituted breast milk PCBs, respectively. The data show that the ED_{50} values for Kanechlor 500 and the reconstituted breast milk PCBs were approximately 87 and 12 $\mu mol \cdot kg^{-1}$, respectively; the reconstituted breast milk PCB mixture, therefore was about seven times more biologically active than the commercial mixture.

The increased potency of the breast milk PCB mixture reflects the preferential bioconcentration of 2,3',4,4',5-penta-, 2,3,3',4,4'-penta- and 2,3,3',4,4',5-hexachlorobiphenyl which have recently been shown to be potent mixed-type inducers (19). Furthermore, each of these three mixed-type inducers has been shown to elicit various toxic responses in the rat (16,17,21) or chicken (5). The bioconcentration of these toxic PCB congeners in breast milk suggests that regulatory agencies should reassess allowable daily intake (ADI) values for PCBs. Current ADI values have been based on the toxicity of the commercial PCBs and not on the toxicity of the PCB mixtures which are actually consumed.

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