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Lymphoproliferative Responses of Splenocytes from Wild Cotton Rats (*Sigmodon hispidus*) Following Acute Exposure to Aroclor 1254

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Polychlorinated biphenyls (PCBs) as environmental contaminants are known for their toxicity, persistence, and relative abundance throughout the world (McFarland and Clarke 1989). Animal experiments have shown that chronic exposure to polychlorinated biphenyls can compromise immune function (Tryphonas 1994). In fact, studies of laboratory animals have confirmed that toxicity of PCBs may in large part be due to the sensitivity of the immune system to these chemicals (Tryphonas 1995). Mechanisms of PCB-induced immunotoxicity are not fully elucidated but have been linked to binding of certain coplanar congeners to aryl hydrocarbon (Ah) receptors in similar fashion to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Safe 1984; Silkworth et al. 1984). Other possible mechanisms of action include the metabolic conversion of certain congeners to arene-oxide intermediates that may covalently bind cellular macromolecules disrupting enzyme function (Tryphonas 1995), and less well elucidated non-dioxin-like mechanisms (Giesy and Kannan, 1998). Commercial mixtures of PCB congeners like Aroclor 1254 contain both Ah-receptor binding congeners, and others with little or no Ah-receptor binding affinity. Toxic equivalents (TEQs) for commercial mixtures of PCBs derived from immunotoxicity-based toxic equivalency factors (TEFs) demonstrate antagonistic or nonadditive interactions among congeners (Harper et al. 1995).

Much more is known regarding the immune response of domesticated and laboratory animals than wildlife species following PCB exposure. Wildlife species are often at high risk to PCB exposure when they inhabit or utilize contaminated sites, with potential exposure through foraging and grooming. Exposure of wildlife species to PCBs could result in immunocompromised individuals, leading to reduced survival and/or fitness of individuals within a population. The cotton rat (*Sigmodon hispidus*) is a wildlife species that has been proposed as a sentinel of environmental contamination (Elangbam et al. 1989; McMurry et al. 1994). Cotton rats are abundant throughout much of the Southeastern United States in numerous habitat types, and are commonly found on hazardous waste sites (Rattner et al. 1993; Flickinger and Nichols 1990). This study was designed to examine the sensitivity of selected endpoints of immunity in wild cotton rats exposed to PCBs.

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MATERIALS AND METHODS

Twenty cotton rats (14 male and 6 female) representing four litters from an outbred colony at Oklahoma State University, Stillwater, Oklahoma, USA were used in this experiment. Animals were between 90 to 150-days of age with a mean (±SE) body mass of 157 ± 7g. Cotton rats were housed individually in polystyrene cages (43x27x19cm) with wire lids and pine chip bedding. All animals were maintained on a 12:12 hour light:dark cycle and provided food (Purina Rodent Chow, Purina 5001, St. Louis, MO, USA) and water *ad libitum*.

An individual from each litter was assigned to each of five treatment groups. All PCB treatment groups consisted of four males, whereas positive and negative control groups each contained three females and one male. Treatment groups included three PCB dose levels (15, 60, or 250mg/kg body mass Aroclor 1254), cyclophosphamide (CY, 50mg/kg) as an positive control, and sterile filtered corn oil vehicle as a negative control, administered intraperitoneally for three consecutive days to simulate extensive acute exposure. Cotton rats were estimated to weigh 200g. Actual dose levels were later calculated based on exact body weights.

Cotton rats were anesthetized by Metafane inhalation (Methoxyflurane) two days after the last dosage. Approximately 1ml whole blood was collected from the retro-orbital sinus plexus using heparinized micro-hematocrit tubes (75 x 1.1mm id) as a conduit into 3ml hematology vials containing EDTA. White blood cell counts were performed manually using a hemocytometer.

Cotton rats were euthanized by cervical dislocation while under anesthesia and weighed to the nearest ±0.1g. Procedures for culturing splenocytes followed those described by Lochmiller et al. (1993). Briefly, spleens were aseptically removed, weighed to the nearest 0.1mg, and gently disrupted in ground glass tissue homogenizers containing 5ml RPMI-S. RPMI-S consisted of RPMI 1640 tissue culture media supplemented with L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin, 2-mercaptoethanol, and normal horse serum. Cells were allowed to settle and the supernatant decanted into centrifuge tubes. Tubes were centrifuged at 275xg for 7 minutes at 10°C. Resulting pellets were resuspended in 5ml RPMI-S and rewashed twice. Viable cells were counted on a hemocytometer after lysing erythrocytes with tris-buffered 0.83% ammonium chloride and staining with Trypan blue.

Lymphocyte proliferation was assessed by stimulating DUPLICATE90µl aliquots of splenocyte suspensions (500,000 cells) with 10µl of mitogen in 96 well flat-bottom microtiter plates. Mitogens included concanavolin A (Con A, *Canvalia ensiformis*, Sigma Chemical Co., St. Louis, MO, USA, 2.5, 5, 10, and 20µg/ml of culture) and pokeweed mitogen (PWM, *Phytolacca aamericana*, Sigma 0.156, 0.313, 0.625, and 1.25µg/ml of culture). Cultures were incubated at 37°C for 72hr in a humidified incubator with 5% CO_2. Three hours before terminating

incubation, 10µl of MTT (tetrazolium salt, 5mg/ml in PBS; Sigma) were added to each well. At 72hr, incubation was terminated by adding 160µl of acid-isopropanol (176µl concentrated HCL and 49.824ml isopropanol) to each well and mixing. Absorbances were read on a Titertek Multiscan Plus MK II (Flow Laboratories, McLean, VA, USA) at 570 and 640nm and compared to unstimulated cultures.

Net absorbances (absorbance of culture – absorbance of blank) are reported for all treatment groups. All data were rank-transformed due to heterogeneity of variances and differences in mass and immune responsiveness were determined by one-way analysis of variance (PROC GLM, SAS Institute 1985). Differences in spleen weight and splenic cellularity were examined using analysis of covariance (PROC GLM, SAS Institute 1985) using body mass and spleen mass as covariants, respectively. When significance was determined, Duncan's multiple range test was used for comparisons among treatment means when main effects were different. Single degree of freedom linear contrasts were used to assess main effects (PCB versus negative controls). Differences were deemed significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Twelve male animals were dosed with PCBs and three females and one male were dosed with CY based on an estimated body mass of 200g. However, actual body mass of all animals was less than 200g, resulting in dose levels higher than prescribed in the initial experimental design. Actual mean dose levels (mg/kg body mass $\pm$SE) for cotton rats were 19$\pm$1 for the low (15) PCB group, 70$\pm$4 for the medium (60) PCB group, 314$\pm$30 for the high (250) PCB group, and 74$\pm$15 for the CY group.

We observed no gross behavioral changes or tissue pathology in any animals during the study. Spleen mass ($P=0.1068$) and total splenocytes ($P=0.3389$) were not significantly different among treatments, but varied considerably among individuals (Table 1). Spleen mass and cellularity were lowest in CY-treated cotton rats. Total splenocyte counts for cotton rats dosed with CY did not differ from negative control animals, although their mean count was only 58% of the control. Total splenocyte counts for cotton rats in the medium PCB group were approximately 60 to 70% greater than animals in the low PCB and negative control groups. White blood cell counts differed significantly among treatments ($P=0.0069$, Table 1). However, effects were due solely to CY-induced reductions in white cell numbers ($P=0.0273$), with WBC counts in cotton rats dosed with CY approximately 50% of negative controls. No differences in WBC counts were observed between PCB-dosed cotton rats and negative control animals ($P>0.05$).

Proliferation of splenocytes varied significantly across treatments in some cultures stimulated with Con A and PWM (Fig. 1). Proliferative response to 2.5 and 5.0 and 20µg of Con A was similar ($P>0.05$) across all treatment groups.

Conversely, splenocytes cultured with 10μg Con A differed significantly (P=0.0240) among treatments with negative control animals lower than all others except the CY-treated group. Specific contrasts indicated that cotton rats dosed with PCBs had significantly higher lymphoproliferative responses than negative controls in cultures stimulated with 10.0 (P=0.0025) and 20μg Con A (P=0.0152). Similarly, cotton rats dosed with CY had higher proliferative responses than negative controls in cultures stimulated with 20.0μg Con A (P=0.0077).

Table 1. Mean (SE) dose, body and organ mass, and cellularity of control cotton rats and those exposed to cyclophosphamide (CY), and three (low, meduim, high) dosages of Arochlor 1254.

	Treatment				
Parameter	CY (n=4)	Control (n=4)	Low (n=4)	Med (n=4)	High (n=4)
Spleen mass (mg)	86.6(10.2)	104.5(11.9)	117.6(13.3)	166.4(52.4)	141.9(26.1)
Total splenocytes ($x10^6$)	35.2(11.5)	60.1(14.6)	67.3(12.0)	104.8(43.9)	82.0(20.0)
WBC ($x10^3$ cell/ml)	2.4(0.5)a	5.0(0.6)b	4.9(0.9)b	6.7(0.6)b	5.8(0.2)b

a,b indicates significant differences (P<0.05)

Proliferative responses of splenocyte cultures stimulated with 0.313, 0.625, and 1.25μg PWM were similar across all treatment groups (Fig. 1). However, 0.156μg PWM elicited significantly different proliferation responses among treatment groups (P=0.0279). Negative control splenocytes had higher proliferative responses than the high PCB and the CY-treated groups. Low PCB group splenocytes also had higher proliferative responses than the CY-treated group. Specific contrasts indicated that cotton rats dosed with corn oil only had significantly higher lymphoproliferative responses than the CY-treated group in cultures stimulated with 0.156 (P=0.0031) and 0.313μg PWM (P=0.0338).

Spleen mass was unaffected by PCB treatment or CY in this study. Similarly, Smialowicz et al. (1989) treated male weanling Fischer 344 rats with Aroclor 1254 by gastric intubation at doses up to 125mg/kg body mass/day for 15 weeks and saw no changes in spleen weights. Male BALB/c mice dosed with 167 PPM of Aroclor 1242 in their diet had no changes in splenic mass (Loose et al., 1977). However, C57BL/6 mice dosed with 350 μmol/kg of the mono-ortho substituted congener 2,3,3',4,4',5- hexachlorobiphenyl had reduced relative spleen weights in another study (Silkworth et al. 1984).

Total harvested splenocytes were not significantly different among treatment groups although splenic cellularity appeared to be elevated in the medium PCB dosed cotton rat group. Wierda et al. (1981) exposed C57BL/6 mice to Aroclor 1254 by IP injection on four occasions at doses ranging from 63 to 550mg/kg and found reduced splenic cellularity. Sample size limitations in the present study

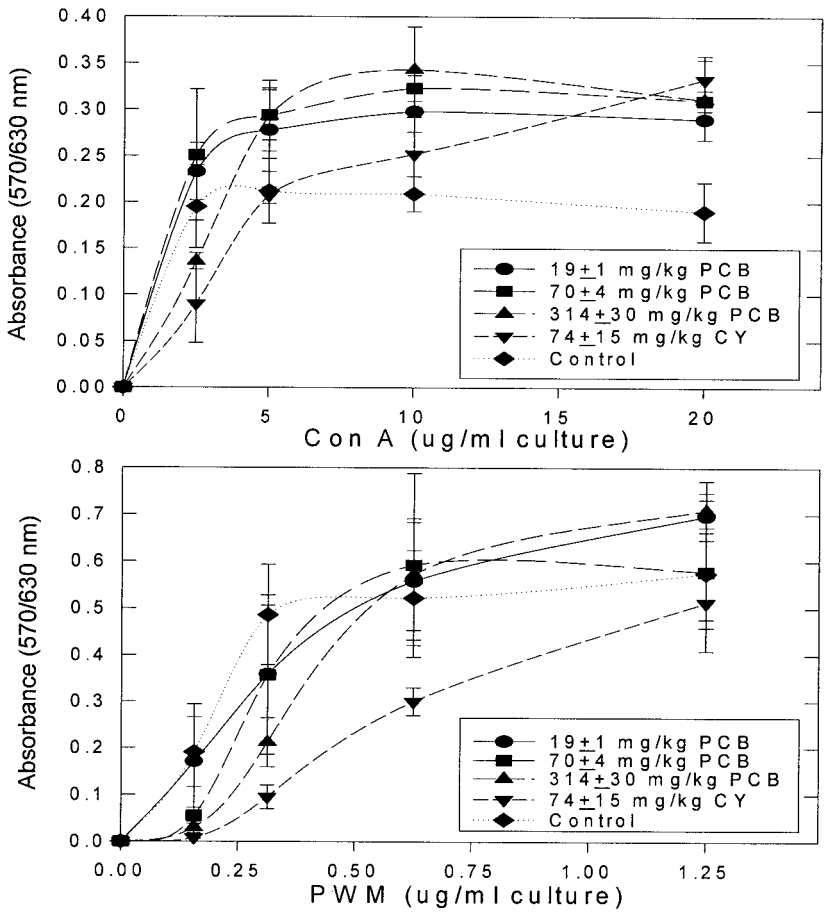

Figure 1. Mean (±SE) absorbance values of splenocyte cultures following *in vitro* stimulation with four concentrations each of Concanavolin A (Con A) (2.5, 5.0, 10.0, and 20.0 µg/ml culture) and Pokeweed mitogen (PWM) (0.156, 0.313, 0.625, and 1.25µg/ml culture) in cotton rats administered 19±1, 70±4, or 314±30 mg/kg body mass (mean±SE) Aroclor 1254, 74±15 mg/kg body mass cyclophosphamide (CY) as positive control, and sterile filtered corn oil vehicle as a negative control IP for three consecutive days. Sample size = 4 in all cases.

may have reduced our ability to detect significant alterations in splenocyte numbers, especially in the CY-treated cotton rats.

White blood cell counts were unaffected by PCB treatment although cyclophosphamide-treated rats had significantly reduced WBC counts compared to negative controls. Cyclophosphamide is an immunosuppressive drug that selectively kills actively dividing cells such as proliferating lymphocytes *in vivo* (Janeway and Travers 1996). B-cells appear to be more sensitive to CY-induced mitotic inhibition than T-cells at low dosages (Misra and Bloom 1991).

Mitogenic stimulation with Con A (10 and 20μg/ml) produced greater lymphoproliferative responses in the PCB treated rat cultures than controls (Fig. 1.) Con A is primarily a T-cell mitogen, indicating PCB-modulated changes in T-cell proliferative potential. Our results are similar to those of Lubet and coworkers (1986) who saw no depression, and a slight but significant increase in lymphoproliferative response of splenocytes from mice exposed to Aroclor 1254. Harbour seals (*Phoca vitiulina*) chronically exposed to polyhalogenated aromatic hydrocarbons in fish had declining lymphoproliferative responses attributed to T-cell function when peripheral lymphocyte cultures were stimulated with Con A (Ross et al. 1996). Rhesus monkeys (*Macaca mulatta*) exposed to Aroclor 1254 orally for 55 months showed declines in lymphoproliferation with Con A that were dose-related (Tryphonas et al. 1991). Lahvis et al. (1995) demonstrated an inverse correlation between Con A-induced lymphoproliferation and tetra- through octa-chlorinatedbiphenyls and other organochlorine compounds in bottlenose dolphins (*Tursiops truncatus*) found along the Atlantic coast. Alternatively, some studies indicate that Con A is not a suitable mitogen for detecting PCB-induced alterations in lymphoproliferative response (Smialowicz et al. 1989, Wierda et al. 1981). Although Aroclor 1254 did not produce changes in mouse lymphoproliferation, it did ameliorate benzene-induced alterations when compared to benzene treated mice in the Wierda et al. (1981) study.

PWM mitogenic stimulation did not elicit significantly different responses between PCB treated and control rat cultures, however differences between negative control cultures and those from CY-treated animals were apparent. This indicates that B-cell function in cotton rats may not be susceptible to short-term acute IP Aroclor 1254 insult. Numerous studies have demonstrated similar results in other mammalian species including Sprague-Dawley rats, C57BL/6 mice, and rhesus monkeys (Bonnyns and Bastomsky 1976; Smialowicz et al. 1989; Tryphonas et al. 1991). However, Ross et al. (1996) found that Con A, PWM, and phytohaemagglutinin were all useful mitogens when assessing polyhalogenated aromatic hydrocarbon-induced lymphoproliferative responses in harbour seal lymphocyte cultures.

The development of the immune system can be age-dependent. Lymphoproliferative response of cotton rats has been shown to vary with age (Lochmiller et al. 1993; McMurry et al. 1994). Additionally, the

immunotoxicological response of mammals to PCB exposure has been shown to vary with age (Lubet et al. 1986). However, all cotton rats used in this study were of similar age, reducing the variability associated with age-dependent immunotoxicity. Gender can also affect toxicological response to PCBs and other Ah-receptor inducing contaminants that are metabolized by P-450 enzymes (Ronis and Cunny 1994), but stimulation indices among male and female cotton rats within treatments groups did not appear different in this study. However, gender-related variation in toxicant metabolism and thus immunotoxicological response may have interfered with our ability to detect differences in proliferative response among treatment groups.

From our findings or altered lymphocyte transformation, we infer that cotton rat immune function may potentially be altered after short-term acute exposure to Aroclor 1254. Con A-stimulated cultures demonstrated differences between PCB dosed animals and controls, but not PWM. Therefore, the PCB-induced immune effects in cotton rats are most likely T-cell linked. Tryphonas (1994) suggests that PCBs may affect only a sub-population of T-cells. Our findings, although equivocal, indicate T-cell involvement in PCB-exposed cotton rats and would support that hypothesis. Additional studies including antigen or microbial challenges would be necessary to more fully determine the immunological significance of acute PCB exposure in cotton rats.

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REFERENCES

Bonnyns M, Bastomsky CH (1976) Polychlorinated biphenyl-induced modification of lymphocyte response to plant mitogens in rats Experientia 32:522-523

Elangbam CS, Qualls Jr CW, Lochmiller RL, Novak J (1989) Development of the cotton rat (Sigmodon hispidus) as a biomonitor of environmental contamination with emphasis on hepatic cytochrome P-450 induction and population characteristics. Bull Environ Contam Toxicol 42:482-488

Flickinger EL, Nichols JD (1990) Small mammal populations at hazardous waste disposal sites near Houston, Texas, USA. Environ Pollut 65:169-180

Giesy JP, 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

Harper N, Connor K, Steinberg M, Safe S (1995) Immunosuppressive activity of polychlorinated biphenyl mixtures and congeners: Nonadditive (antagonistic) interactions. Fund Appl Toxicol 27:131-139

Janeway CA, Jr, Travers P (1996) Immunobiology: The Immune System in Health and Disease. Current Biology Lmt./Garland Publishing Inc., New York, New York

Lahvis GP, Wells RS, Kuehl DW, Stewart JL, Rhinehart HL, Via CS (1995) Decreased lymphocyte responses in free-ranging bottlenose dolphins (Tursiops truncatus) are associated with increased concentrations of PCBs and DDT in peripheral blood. Environ Health Perspect 103:67-72

Lochmiller RL, Vesty MR, McMurry ST (1993) Phenotypic variation in lymphoproliferative responsiveness to mitogenic stimulation in cotton rats. J Mammal 74:189-197

Loose LD, Pittman KA, Benitz KF, Silkworth JB (1997) Polychlorinated biphenyl and hexachlorobenzene induced humoral immunosuppression. J Reticuloendothelial Soc 22:253-71

Lubet RA, Lemaire BN, Avery D, Kouri RE (1986) Induction of immunotoxicity in mice by polyhalogenated biphenyls. Arch Toxicol 59:71-77

McFarland VA, Clarke JU. 1989. Environmental occurance, abundance, and potential toxicity of polychlorinated biphenyl congeners: Considerations for a congener-specific analysis. Environ Health Perspect 81:225-239

McMurry ST, Lochmiller RL, Vesty MR, Qualls CW, Jr (1994) Immunological responses of weanling cotton rats (Sigmodon hispidus) to acute benzene and cyclophosphamide exposure. Bull Environ Contam Toxicol 52:155-162

Misra RR, Bloom SE (1991) Roles of dosage, pharmacokinetics, and cellular sensitivity to damage in the selective toxicity of cyclophosphamide towards B and T cells in development. Toxicology 66:239-256

Rattner BA, Flickinger EL, Hoffman DJ (1993) Morphological, biochemical, and histopathological indices and contaminant burdens of cotton rats (Sigmodon hispidus) at three hazardous waste sites near Houston, Texas, USA. Environ Pollut 79:85-93

Ronis MJJ, Cunny HC (1994) Physiological (endogenous) factors affecting the metabolism of xenobiotics. In: Hodgson E, Levi PE (eds) Introduction to Biochemical Toxicology, Second Edition. Appleton & Lange: Norwalk, Connecticut

Ross P, DeSwart R, Addison R, VanLoveren H, Vos J, Osterhaus A (1996) Contaminant-induced immunotoxicity in harbour seals: Wildlife at risk? Toxicology 112:157-169

SAS Institute Inc. (1985) SAS user's guide:statistics, version 5 edition. Cary, North Carolina: SAS Institute Inc.

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

Silkworth JB, Antrim L, Kaminsky LS (1984) Correlations between polychlorinated biphenyl immunotoxicity, the aromatic hydrocarbon locus, and liver microsomal enzyme induction in C57BL/6 and DBA/2 mice. Toxicol Appl Pharm 75:156-165

Smialowicz RJ, Andrews JE, Riddle MM, Rogers RR, Leubke RW, Copeland CB (1989) Evaluation of the immunotoxicity of low level PCB exposure in the rat. Toxicology 56:197-211

Tryphonas H (1994) Immunotoxicity of polychlorinated biphenyls: Present status and future considerations. Exp Clin Immunogenet 11:149-162.

Tryphonas H (1995) Immunotoxicity of PCBs (Arochlors) in relation to Great Lakes. Environ Health Perspect 103(suppl. 9):35-46

Tryphonas H, Luster MI, Schiffman G, Dawson LL, Hodgen M, Germolec D, Hayward S, Bryce F, Loo JCK, Mandy F, Arnold DL (1991) Effect of chronic exposure of PCB (Arochlor 1254) on specific and nonspecific immune parameters in the Rhesus (*Macaca mulatta*) monkey. Fund Appl Toxicol 16:773-786

Wierda D, Irons RD, Greenlee WF (1981) Immunotoxicity in C57BL/6 mice exposed to benzene and Arochlor 1254. Toxicol Appl Pharm 60:410-417