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Lymphoma and Lung Cancer in Offspring Born to Pregnant Mice Dosed with Dibenzo[*a,l*]pyrene: The Importance of *In Utero* Versus Lactational Exposure

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Abstract

The fetus and neonate cannot be viewed as “little adults”; they are highly sensitive to toxicity from environmental chemicals. This phenomenon contributes to the fetal basis of adult disease. One example is transplacental carcinogenesis. Animal models demonstrate that environmental chemicals, to which pregnant women are daily exposed, can increase susceptibility of the offspring to cancer. It is uncertain to what degree *in utero* versus lactational exposure contributes to cancer, especially for hydrophobic chemicals such as polyhalogenated biphenyls, ethers, dioxins, furans, etc., which can partition into breast milk. We developed a pregnant mouse model in which exposure to the polycyclic aromatic hydrocarbon (PAH), dibenzo[*a,l*]pyrene (DBP), during late gestation, produces an aggressive T-cell lymphoma in offspring between 3-6 months of age. Survivors exhibit multiple lung and liver (males) tumors. Here, we adopt a cross-foster design with litters born to dams treated with DBP exchanged with those born to dams treated with vehicle. Exposure to DBP *in utero* (about 2 days) produced significantly greater mortality than residual DBP exposure only through breast milk (3 weeks of lactation). As previously observed pups in all groups with an *ahr*^{*b-1/d*} (“responsive”) genotype were more susceptible to lymphoma mortality than *ahr*^{*d/d*} (“non-responsive”) siblings. At termination of the study at 10 months, mice exposed *in utero* also had greater lung tumor multiplicity than mice exposed only during lactation. Our results demonstrate that short exposure to DBP during late gestation presents a greater risk to offspring than exposure to this very hydrophobic PAH following 3 weeks of nursing.

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Keywords

Transplacental cancer; PAHs; lymphoma; lung cancer; cross-foster

INTRODUCTION

The fetus and neonate are exposed to a number of toxic chemicals in the environment through a variety of exposure routes, often dietary (Autrup, 1993; Birnbaum, 1994; Fenster *et al.*, 2006; Landrigan *et al.*, 2002; Massart *et al.*, 2005; McLachlan, 1993; Perera *et al.*, 2003; 2006; Saso and Vainio, 1999; Schnaas *et al.*, 2006; Schonfelder *et al.*, 2002; Smith *et al.*, 2006; Wang *et al.*, 2005). It has been recognized for a number of years that the fetus and neonate exhibit increased sensitivity to a number of these environmental chemicals (Landrigan *et al.*, 2004). Regulatory agencies have adopted the policy that a child is not a “little adult” and most acceptable exposure levels incorporate a safety factor to specifically recognize that fact (Landrigan *et al.*, 2004). The study of epigenetics and imprinting has shown that exposure to agents during early stages of development can program gene expression such that susceptibility to disease in later life is impacted (Ho and Tang, 2007; Prins *et al.*, 2008).

A number of animal models of transplacental cancer have been developed (Anderson, 2004a; 2004b; Liu *et al.*, 2007; Miller, 2004; Miller *et al.*, 2000). Epidemiology studies in human populations find high exposures to environmental chemicals associated with impacts on offspring including birth weight, behavioral endpoints and diseases including cancer, although not all studies show a positive correlation (Autrup, 1993; Birnbaum, 1994; Dallaire *et al.*, 2006; Eskenazi *et al.*, 2003; Fenster *et al.*, 2006; Jacobson *et al.*, 2002; Leem *et al.*, 2006; McLachlan, 1993; Massart *et al.*, 2005; Miller *et al.*, 2004; Perera *et al.*, 2003; 2006; Saso and Vainio, 1999; Schnaas *et al.*, 2006; Schonfelder *et al.*, 2002; Smith *et al.*, 2006; Soechitram *et al.*, 2004; Wang *et al.*, 2005; Whyatt *et al.*, 2001; 2002; 2004; Wild and Kleinjans, 2003;).

Exposure to environmental carcinogens will occur *in utero* if the chemical can effectively cross the placenta and during nursing if capable of partitioning into breast milk. The beneficial effects of breast feeding for infants have been repeatedly demonstrated (Turck, 2005). Still, many women worry about chemical exposure to their nursing infants given the number of chemicals, including polyhalogenated biphenyls, dioxins, ethers, dibenzofurans, etc., measured in breast milk; the levels of some being positively correlated to adverse impacts in children (Landrigan *et al.*, 2002; Massart *et al.*, 2005; McLachlan, 1993;).

Our laboratory has developed a mouse model of transplacental cancer with the potent polycyclic aromatic hydrocarbon (PAH), dibenzo[*a,l*]pyrene (DBP). Administration of DBP during late gestation produces a high rate of mortality in the offspring from an aggressive T-cell lymphoma (Yu *et al.*, 2006a). If the offspring do not succumb to the lymphoma, 100% have multiple lung tumors and most males liver lesions. We developed this model as a potential novel approach to dietary chemoprevention. Inclusion of known cancer chemopreventive agents in the maternal diet (e.g., indole-3-carbinol (I3C) or green tea) or co-administration with chlorophyllin markedly reduced lymphoma mortality and/or lung cancer, even though the offspring were not exposed to these chemopreventive agents in their diet (Castro *et al.*, 2008b; Yu *et al.*, 2006b).

In further characterization of this model, we felt it important to address the question of how much of the cancer in the offspring was due to DBP bioavailability and bioactivation in fetal target organs during the 2 days of *in utero* exposure compared to neonatal exposure and bioactivation during 3 weeks of nursing. This information is important in estimating risk of

maternal diets and supplements and drug or xenobiotic exposure during nursing and in design of optimal chemoprevention strategies.

We report here that the brief *in utero* exposure contributed to a greater degree than residual DBP partitioning into breast milk. This observation was accomplished by switching the litters at birth so that they were exposed only *in utero* or only during breast feeding. We also included a group where the litters were not cross-fostered to compare the results to offspring that could accumulate the PAH through both routes of exposure. These results applied to both DBP-dependent lymphoma mortality and to lung tumor multiplicity and highlight the importance of reducing exposures as much as possible to toxic chemicals in the environment during pregnancy and to the potential for supplementation of the maternal diet with cancer chemoprotective agents.

METHODS

Chemicals and diets

DBP was obtained from the NCI carcinogen repository at the Midwest Research Institute (Kansas City, MO) and was at least 98% pure as determined by HPLC. The semipurified diets, AIN93G and AIN93M, were purchased from Research Diets (New Brunswick, NJ). Ethidium bromide was purchased from Sigma Chemical Co. (St. Louis, MO). Direct PCR lysis reagent and proteinase K was purchased by Viagen Biotech Inc. (Los Angeles, CA). Taq PCR master mix was purchased from Promega Co. (Madison, WI) and Novex TBE Gels were purchased from Invitrogen Technologies (Carlsbad, CA).

Animals and treatment protocols

Eight-week old B6129SF1 female and 129S1/SvImJ male mice (Jackson Laboratories, Bar Harbor, ME) were housed at the Laboratory Animal Resource Center at Oregon State University. The mice were maintained in a pathogen free environment at $20 \pm 1^\circ \text{C}$ and $50 \pm 10\%$ humidity, with a light dark cycle of 12 hours in micro-isolator cages (Life Products, Inc., Seaford, DE) with CareFRESH bedding. During breeding, gestation, and lactation mice were allowed free access to AIN93G diet. After acclimation for one week, mice were bred and appearance of the vaginal plug determined to be day 0. Pregnant mothers were treated on the 17th day of gestation by oral gavage with either vehicle (corn oil, 5 mL/kg body weight) or 15 mg/kg DBP in the same volume of corn oil. On the first day following parturition, a litter born to a dam receiving DBP was cross-fostered with a litter born to a mother receiving corn oil vehicle. Another subset of litters were not cross-fostered and represented either total DBP exposure (transplacental and lactational) or vehicle control. Figure 1 details the group assignments, experimental timeline, as well as the number of offspring in each experimental group. Upon weaning, mice remained on AIN93G diet until switching to AIN93M at 3 months of age. If any signs of distress or pain (or any sign of morbidity) were observed, mice were immediately euthanized with an overdose of CO_2 and a necropsy performed. No lymphomas were observed in offspring that were born to mothers receiving vehicle alone (data not shown). At 10 months of age, the study was terminated and any surviving mice euthanized and necropsied. All procedures used in the handling, treatment and husbandry of mice were approved by the Oregon State University Institutional Animal Care and Use Committee.

Histopathology

Upon necropsy, heart, thymus, lung, spleen, liver, kidney, abnormal lymph nodes, testes or ovaries, colon and skin were fixed in 10% formalin, stained with hematoxylin and eosin and analyzed as described previously (Castro et al., 2008a;2008b; Yu et al., 2006a;2006b). The incidence of lymphoma leading to morbidity as well as lung lesions were scored by gross necropsy and a sub-set from each group confirmed by histopathology (Supplement Table).

Genotyping for aryl hydrocarbon receptor (*ahr*) b-1 and d alleles

We previously demonstrated that the mouse *ahr* genotype influenced offspring susceptibility to lymphoma mortality (Yu et al., 2006a). Our breeding scheme was designed such that, on average, litters should be comprised of half *ahr*^{b-1/d} and half *ahr*^{d/d} siblings. At necropsy, an 8 mm ear-punch was collected and lysed overnight at 55°C in 100 µl of DirectPCR Lysis Reagent containing proteinase K, followed by 45 min at 85°C. The resulting lysate was briefly centrifuged prior to undergoing a PCR reaction with allele-specific primers to permit one-tube genotyping of the *ahr* alleles as previously described (Yu et al., 2006a). PCR products were separated and visualized on Novex 8% Tris-borate EDTA gels. Ahr responsiveness (*ahr*^{b-1/d}) was confirmed by the presence of two PCR products of 159 bp (*ahr*^{b-1}) and 148 bp (*ahr*^d). Non-responsive mice had a single product of 148 bp. A molecular weight ladder of *MspI* cut pBR322 DNA and ethidium bromide was used to stain the DNA, followed by UV visualization.

Statistical analysis

The experimental unit for dietary treatments is the dam and offspring responses are, therefore, clustered by litter. Offspring survival curves were compared between treatments using Cox proportional hazards regression and a robust sandwich adjustment to the score test to account for any within-litter dependence. The proportional hazards assumption appeared reasonable based on assessment of Martingale residuals in the SAS phreg procedure (version 9.2).

Offspring tumor multiplicity data were modeled with methods appropriate for counts clustered into litters. Results shown are from a generalized linear mixed model with random litters, log link and negative binomial conditional distribution which was fit with the SAS glimmix procedure (version 9.2). P-values are from an overall model for all survivors. Estimates of multiplicity ratios comparing *ahr* genotypes within each treatment are from separate model fits for each treatment. Very similar results and the same conclusions were reached using two other modeling approaches for counts: (1) Transform response to the square root of the tumor count, then apply linear mixed models with random litters using the SAS mixed procedure and (2) generalized linear mixed model with random litters, log link, Poisson conditional distribution and empirical residual variation (quasilikelihood). All approaches revealed strong evidence of within-litter dependence and the need, therefore, to retain random litters in the models.

The effect of *in utero* DBP on birth weights and litter size was investigated by comparing the lactational litters (no DBP *in utero*) to the litters from the other two treatments (both of which received DBP *in utero*). For per offspring birth weights linear mixed models with random litters was used (SAS mixed procedure). For per dam litter sizes one-way ANOVA was used.

RESULTS AND DISCUSSION

The experimental design of the cross-foster study resulted in 4 groups of offspring and a total of 215 mice. The timeline of exposure as well as the number of dams and offspring used for each experimental group is given in Figure 1. As seen previously (Yu et al., 2006a; Yu et al., 2006b; Castro et al., 2008a; 2008b), treatment of pregnant mice with DBP did not elicit acute maternal or fetal toxicities; there was also no evidence of reduction in litter size or birth weight ($p > 0.4$ for all comparisons, data not shown). Offspring born to dams treated with DBP exhibited the first mortalities at about 6 weeks of age but, as previously observed (Yu et al., 2006a), the rate of mortality increased markedly between 12 and 24 weeks of age (Figure 2). In litters where offspring exposed to DBP *in utero* were switched at birth and nursed by dams treated only with vehicle, the survival curves demonstrated significantly less ($p = 0.0133$) mortality than pups in litters nursed by their own mother. There were surprisingly few DBP-dependent mortalities in litters born to control mothers that we cross-fostered to mothers that had been

treated with DBP. These results suggest that only a small portion of the lymphoma risk was from DBP exposure in breast milk (pups exposed *in utero* only had significantly lower survival ($p=0.0087$) than pups exposed only during nursing). It is possible that a relatively small amount of the DBP partitions into breast milk. If the DBP is eliminated fairly rapidly from maternal compartments (e.g., $t_{1/2}$ on the order of hours), there may be little left to be available to the pups through nursing. We are conducting further studies employing ^{14}C -DBP to address these pharmacokinetic issues.

In this model of transplacental PAH-dependent cancer, we utilized a C57B6129F1 female back-crossed to a 129 male. This experimental design allowed us to examine the role of the fetal *ahr* genotype in tumor response as litters should have equal numbers of Ahr responsive and non-responsive pups. This allowed us to test hypotheses concerning chemopreventive agents (e.g., I3C) functioning through the Ahr. We previously found a maternal *ahr*^{b-1/d} genotype reduced risk to the fetus (probably through enhanced maternal metabolism and reduced bioavailability to the fetus), but a fetal *ahr*^{b-1/d} genotype increased the risk of to the offspring irrespective of the mother's genotype (Yu *et al.*, 2006a; 2006b). In this study, we again found a responsive fetal *ahr* genotype reduced the lymphoma survival in all groups (offspring exposed *in utero* and lactationally, 19.2 VS 32%; *in utero* only, 50 VS 70.4%; and lactationally only, 86.7 VS 96.2%, respectively; Table 1). If the lymphoma survival curves shown in Figure 2 were plotted for each genotype, it would clearly demonstrate that *ahr*-responsive pups in all three groups have reduced lymphoma survival ($p=0.0217$ using a Cox proportional hazards regression with treatment as strata and with a robust test score to adjust for litters; data not shown). The thymus (mouse and human) expresses high levels of Cytochrome P450 (Cyp) 1b1 in late gestation and shortly following parturition (Choudhary *et al.*, 2003; 2005). Cyp1b1 has the greatest activity of any CYP in bioactivation of DBP to carcinogenic metabolites, particularly DBP-(-)-*anti*-(11R,12S,13S,14R)-dihydrodiol epoxide (Luch *et al.*, 1998; Shimada and Fujii-Kuriyama, 2004). In an earlier publication, employing *cyp1b1* knockout mice, we demonstrated a strong gene dosage effect. Cyp1b1 null offspring were completely resistant, wild-type siblings sensitive and the hets intermediate with respect to lymphoma-dependent mortality (Castro *et al.*, 2008a). In this study, we do not see complete resistance in offspring with the *ahr*^{d/d} genotype. The *ahr*^d allele is still capable of induction by potent Ahr ligands or by higher doses of ligands with more modest binding affinities. DBP may function in either of these modes and induce Cyp1b1 to some degree in the *ahr*^{d/d} offspring. Alternatively, these "non-responsive" mice may exhibit some lymphoma-dependent mortality due to bioactivation by other CYPs, perhaps with contributions from peroxidases (Cavaliere and Rogan, 1990) and/or aldo-keto reductases (Palackal *et al.*, 2001). Fetal Cyp1b1 expression in thymus may also be controlled by an AhR-independent mechanism, perhaps involving methylation of the *cyp1b1* promoter region (Nakajima *et al.*, 2003; Tokizane *et al.* 2005). These potential mechanisms require further study to assess their contribution to DBP carcinogenesis in this model.

Surviving offspring born to dams treated with DBP at 15 mg/Kg exhibit a 100% incidence of lung tumors with a multiplicity per tumor bearing animal of approximately 15_[P1]. As with the lymphoma mortality, offspring exposed to DBP only *in utero* exhibited greater lung tumor multiplicity than offspring in litters that were exposed to DBP only through nursing (Table 1). When exposure was *in utero*, the surviving offspring that were Ahr "responsive" had significantly more lung tumors than their "non-responsive" siblings ($p<0.002$). However, the genotype difference was not as apparent with litters exposed to DBP only through nursing, 3.3 and 2.6 for responsive and non-responsive offspring ($p>0.5$). One possible explanation is that DBP is bioactivated in the lung more efficiently by Cyp1a1 which is induced to a greater degree through the *ahr* than Cyp1b1. If the reduced lung tumor multiplicity in the lactational only exposure is caused by a lower dose to the infant due to high maternal clearance, this would

also explain the lack of impact of ahr signaling. Another possibility would be increased expression of phase II detoxication enzymes following parturition in the lung.

PAHs represent a “re-emerging” environmental concern. Due to high energy demands, the excess burning of coal and petroleum is increasing the emission of PAHs. For example, 70% of the electricity generated in China and 50% in the U.S., comes from coal burning (Xu *et al.*, 2006). These two countries are also the two largest automobile markets. Particulate matter (PMs), containing PAHs, reach the U.S. west coast from China in less than a week. As women of child-bearing age will be increasingly exposed, we chose PAHs to study in our model of transplacental cancer.

Cancer is the number 1 cause of disease-related death in children (2nd overall, after accidents) and lymphomas/leukemias are the most common of these cancers (Anderson *et al.*, 2000). According to the Leukemia and Lymphoma Society, over 800,000 Americans have leukemia, lymphoma or myeloma with an estimated 135,500 new cases and over 50,000 deaths annually (<http://www.leukemia-lymphoma.org>). Lung cancer is still the number one cause of cancer mortality in the U.S. with an estimated 213,380 new cases in 2007 and 160,000 deaths (Jemal *et al.*, 2007). The poor survival rate (15%) in lung cancer patients reinforces the need to develop effective prevention strategies.

Our transplacental model of a PAH-dependent T-cell lymphoma and lung cancer, demonstrating that a brief exposure *in utero* is primarily responsible for a robust response, is remarkable given that the cancers occur in what would be the equivalent in humans of young adults (lymphoma) or middle-age (lung cancer). Furthermore, we have developed effective chemopreventive strategies in this model by supplementation of maternal diet (Castro *et al.*, 2008b; Yu *et al.*, 2006b). In order to devise the most efficacious approaches to chemoprevention, we need to know when the greatest period of sensitivity is. We now show that, in this model, *in utero* exposure carries the greatest risk. This may not be true for carcinogens with different pharmacokinetic properties or pathways of bioactivation. Our long-term goal is to translate this work into potential strategies for providing maximum protection to the fetus from transplacental carcinogens by supplementation or optimizing the maternal diet.

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Figure 1. Experimental Design of DBP exposure in offspring

Pregnant mice were administered DBP or corn oil vehicle at GD17. Offspring were cross-fostered within 12 h of birth and consequently exposed to DBP (diagonal bars) via placenta (group 3) and/or via nursing (groups 2 and 4). Number of dams are indicated in parenthesis.

Figure 2. Survival curves for DBP-dependent lymphoma morbidity in offspring born to dams treated with 15 mg/Kg at gestation day 17

The litters either remained with their mother during nursing (▼) or were cross fostered. Some litters born to mothers dosed with DBP were immediately transferred to mothers treated with vehicle only (□, *in utero* exposure only); conversely, some litters were taken immediately after birth from mothers treated with vehicle and transferred to DBP-treated mothers (▲, lactational only) for the entire 3 weeks of nursing. There is strong statistical evidence of treatment differences ($p=0.009$ for lactational only vs. *in utero* and $p=0.013$ for *in utero* only vs. both using Cox regression with a robust score test).

TABLE 1
Influence of genotype on offspring survival and lung tumor multiplicity in 10 month survivors

Group	% Survival			Lung Tumor Multiplicity			Multiplicity Ratio ^J
	Overall	b ⁻¹ /d	d/d	Overall	b ⁻¹ /d	d/d	
Both	25.5	19.2	32.0	14.5 ± 2.6	15.2 ± 4.7	14.1 ± 3.0	1.12 (0.66, 1.92)
<i>In Utero</i>	60.0	50.0	70.4	11.5 ± 1.1	15.7 ± 1.6	8.2 ± 0.9	1.54 (1.20, 1.97)
Lactational	91.1	86.7	96.2	3.0 ± 0.3	3.3 ± 0.5	2.6 ± 0.3	1.06 (0.69, 1.64)

^JThe multiplicity ratio is estimated from a model that accounts for litters (see Methods)