In utero Exposure of Mice to Dibenzo[a,l]Pyrene Produces Lymphoma in the Offspring: Role of the Aryl Hydrocarbon Receptor

Zhen Yu,1,2 Christiane V. Loehr,3,4 Kay A. Fischer,3,4 Mandy A. Louderback,3 Sharon K. Krueger,1,2 Roderick H. Dashwood,1,2,4 Nancy I. Kerkvliet,4 Clifford B. Pereira,4 Jamie E. Jennings-Gee,6 Stephanie T. Dance,6 Mark Steven Miller,6 George S. Bailey,1,2,4 and David E. Williams1,2,4

1Department of Environmental and Molecular Toxicology, The Linus Pauling Institute, College of Veterinary Medicine, Environmental Health Sciences Center, 2Department of Statistics, Oregon State University, Corvallis, Oregon and 6Department of Cancer Biology, Wake Forest University School of Medicine, Winston Salem, North Carolina

Abstract

Lymphoma and leukemia are the most common cancers in children and young adults; in utero carcinogen exposure may contribute to the etiology of these cancers. A polycyclic aromatic hydrocarbon (PAH), dibenzo[a,l]pyrene (DBP), was given to pregnant mice (15 mg/kg body weight, gavage) on gestation day 17. Significant mortalities in offspring, starting at 12 weeks of age, were observed due to an aggressive T-cell lymphoblastic lymphoma. Lymphocytes invaded numerous tissues. All mice surviving 10 months, exposed in utero to DBP, exhibited lung tumors; some mice also had liver tumors. To assess the role of the aryl hydrocarbon receptor (AHR) in DBP transplacental cancer, B6129SF1/J (AHRb1/d, responsive) mice were crossed with strain 129S1/SvIm (AHRAd/d, nonresponsive) to determine the effect of maternal and fetal AHR status on carcinogenesis. Offspring born to nonresponsive mothers had greater susceptibility to lymphoma, irrespective of offspring phenotype. However, when the mother was responsive, an AHR-responsive phenotype in offspring increased mortality by 2-fold. In DBP-induced lymphomas, no evidence was found for TP53, 5-catenin, or Ki-ras mutations but lung adenomas of mice surviving to 10 months of age had mutations in Ki-ras codons 12 and 13. Lung adenomas exhibited a 50% decrease and a 35-fold increase in expression of Rb and p19/ARF mRNA, respectively. This is the first demonstration that transplacental exposure to an environmental PAH can induce a highly aggressive lymphoma in mice and raises the possibility that PAH exposures to pregnant women could contribute to similar cancers in children and young adults. (Cancer Res 2006; 66(2): 755-62)

Introduction

Lymphoma and leukemia are the most common cancers in U.S. children (1–3). Although childhood cancers represent <1% of all cancers, the 12,400 annual cases result in 2,300 deaths, the greatest cause of disease-related deaths in U.S. children, second only to accidents among all childhood deaths. Approximately 80% to 90% of childhood cancer is of unknown etiology but evidence exists for a contribution from in utero exposure to environmental chemicals including cigarette smoke and pesticides (4–7). However, the evidence is equivocal and only in utero exposures to ionizing radiation and diethylstilbestrol have been definitively linked to enhanced cancer rates in children and young adults (1).

In rodent models, transplacental carcinogenesis has been induced by environmental chemicals such as the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (8), the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (9), and the polycyclic aromatic hydrocarbon (PAH) 3-methylcholanthrene (10–12). The primary targets for these transplacental carcinogens were lung and liver. PAHs are environmental pollutants produced from incomplete combustion of many organic materials including cigarettes, coal, cooking oil, wood, and diesel. The aryl hydrocarbon receptor (AHR), involved in PAH metabolism through induction of cytochromes P450, plays a significant role in tumorigenesis. Different strains of mice exhibit differential sensitivity to environmental carcinogens depending on genetics, such as binding affinity for the AHR and/or allelic variations in loci such as Ki-ras. For example, transplacental administration of 3-methylcholanthrene to backcrosses between B6D2F1 (AHRb1/d, responsive) and D2 (AHRAd/d, nonresponsive) mice results in lung and liver tumors in offspring 1 year after birth (12). An AHR-nonresponsive maternal phenotype enhances risk of carcinogenesis to the fetus. In contrast, if the fetus is AHR responsive, the tumor incidence is higher regardless of the maternal phenotype (10–13). AHR regulates Cyp1a1 and/or Cyp1b1, both of which metabolize 3-methylcholanthrene (and other PAHs) to carcinogenic metabolites (11, 12, 14–18). In the AHR-responsive mother, induction of Cyp1a1 and/or Cyp1b1 is thought to result in enhanced maternal metabolism and reduced bioavailability to the fetus compared with the nonresponsive mother (19, 20). A responsive fetus exhibits enhanced risk for tumor formation independently of the maternal phenotype as induction of Cyp1a1 and/or Cyp1b1 in target organs produces greater bioactivation. In the B6D2F1 × D2 crosses, 3-methylcholanthrene transplacentally induced lung and liver adenomas and carcinomas in the offspring with a high incidence of Ki-ras mutations (21–23). Ki-ras activating mutations have been linked to tumor susceptibility. Allelic polymorphism in Ki-ras confers differential susceptibility to mutation in this gene as a result of metabolism of PAHs. A polymorphism in the second intron of Ki-ras, deletion of a 37-bp repeat, has been linked to lung tumor susceptibility compared with animals without this deletion, which are classified as Ki-ras-resistant strains (Ki-ras- allele; refs. 24, 25).

Dibenzo[a,l]pyrene (DBP) is the most potent PAH carcinogen in fish (26, 27) and rodent models and is a multiorgan carcinogen in
the mouse, producing cancers of the ovary, skin, uterus, and liver, in addition to production of lymphomas (28, 29). DBP is a PAH containing both a bay region and a fjord region. Cytochrome P450 metabolism produces the very potent mutagenic and carcinogenic fjord region metabolite (\(-\)anti-(11R,12S)-dihydrodiol 13S,14R-epoxide \((-\)anti-DBPDE; refs. 30–33\). Studies with knockout mice have shown a crucial role for Cyp1b1 metabolism in DBP (and other PAHs) carcinogenesis (29, 34–36). These results are consistent with studies using expressed human and mouse enzymes, which show Cyp1b1 to have greater activity in generating \((-\)anti-DBPDE, relative to Cyp1a1 or Cyp1a2. Cyp1b1 is abundant in many extra-hepatic tissues of fetal and adult mouse and is regulated in part through the AHR (37–39).

In this study we show, for the first time, that DBP is a transplacental carcinogen in the mouse producing mortalities due to an aggressive thymic lymphoma as early as 3 months of age. Maternal as well as fetal AHR phenotype influenced DBP-transplacental carcinogen in the mouse producing mortalities through the AHR (37–39).

**Materials and Methods**

**Chemicals and diets.** DBP was obtained from the National Cancer Institute chemical carcinogen repository, Midwest Research Institute (Kansas City, MO) at a purity >98% by high-performance liquid chromatography analysis. AIN93G and AIN93M diets were obtained from Dyets, Inc. (Bethlehem, PA).

**Treatment of mice.** Eight-week-old B6129SF1/J and 129S1/SvImJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Laboratory Animal Resource Center at Oregon State University at 20 ± 1°C and 50 ± 10% humidity and a light/dark cycle of 12 hours in micro-isolator cages (Super Mouse 750 Micro-Isolator, Life Products, Inc., Seaford, DE) with CareFRESH bedding. Mice were fed powdered AIN93G diet ad libitum throughout breeding and gestation and offspring fed pelleted AIN93G diet for the first 3 months and continued with AIN93M diet ad libitum until euthanized. Reciprocal crosses between B6129SF1/J and 129S1/SvImJ mice were carried out to produce fetuses gestating in both AHR-responsive and nonresponsive environments. On the 17th day of gestation, pregnant mice were treated with either vehicle (corn oil, 5 mL/kg body weight) or 15 mg/kg body weight DBP in corn oil by gavage. On delivery, the number and sex of live births were recorded. Offspring were nursed for 21 days, weaned onto AIN93G diet, and each sex from the same litter (up to five per cage) housed in micro-isolator cages. Sentinel mice were housed in the same colony and tested for viral or bacterial pathogens and parasites (University of Missouri Research Animal Diagnostic Laboratory, Columbia, MO); all tests were negative. On signs of morbidity, pain, or distress, the mice were euthanized with an overdose of CO2 and necropsied. Surviving mice were euthanized at 10 months of age and necropsied. All procedures for treatment, housing, and euthanasia of the mice used in this study were approved by the Oregon State University Institutional Animal Care and Use Committee.

**Histopathology and immunohistochemistry.** The following tissues were collected at necropsy: heart, thymus, lung, spleen, liver, kidney, abnormal lymph node, testes or ovaries, colon, and skin. Tissues were fixed in 10% formalin, stained with H&E, and analyzed by light microscopy. For determination of the cellular origin of the lymphoma, tumors were stained and analyzed by immunohistochemistry \((n = 20)\) and flow cytometry \((n = 4)\) and found to be T-cell origin. For immunohistochemistry, tissues were sectioned at 4 to 5 µm and placed on Microprobe slides (Fisher Scientific, Pittsburgh, PA). Following rehydration, slides underwent high temperature antigen retrieval using citrate buffer (pH 6.0). DakoCytomation, Carpinteria, CA) for 10 minutes in a microwave pressure cooker (Tendercooker) and placed at room temperature 20 minutes. Slides were washed in Automation buffer (Biomedia, Foster City, CA) followed by blocking in 3% H2O2 in methanol for 10 minutes. After washing in dH2O, slides were placed in the Microprobe capillary gap slide holder (Fisher) and repeatedly washed and blotted to ensure good capillary flow. Slides were blocked first in 1.5% normal goat serum, washed, and blocked with Serum Free Blocking Solution (DakoCytomation) for 10 minutes and blotted. Primary antibody was applied for 30 minutes at room temperature. CD-3 antibody was AlCanbs5600 at a dilution of 1:100 in antibody diluent with background reducing compounds (DakoCytomation). The negative control was Universal Negative Control Rabbit (DakoCytomation). Slides were blotted and washed six times in Automation buffer followed by Envision-horseradish peroxidase rabbit polymer (DakoCytomation) for 45 minutes and again washed and blotted in Automation buffer. The chromagen Nova Red (Vector Laboratories, Burlingame, CA) was applied for 5 minutes. Slides were counterstained in Gill hematoxylin (Shandon, Pittsburgh, PA) followed by 1% lithium carbonate for 30 seconds, cleared in xylene, and coverslipped. The lung tumors were diagnosed as hyperplasia, adenoma, adenoma with progression, and carcinoma (40) and the liver tumors as foci and adenoma.

**Typing of tumor cells by flow cytometry.** Single-cell suspensions of tumor cells were in media supplemented with 10% fetal bovine serum and 15 mmol/L HEPES. Debris was allowed to settle by gravity. Viability of cells was determined microscopically by trypan blue exclusion. Cells were washed and resuspended in PBS containing 1% bovine serum albumin and 0.1% sodium azide. Nonspecific binding of antibodies to Fe receptors was blocked with hamster and rat immunoglobulin G (Jackson ImmunoResearch, West Grove, PA). Cells were stained with fluorochrome-labeled antibodies to CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), and B220 (RA3-682); all purchased from BD Biosciences (San Jose, CA). For some samples, cell viability was determined using propidium iodide. Separate samples were stained with isotype-matched immunoglobulin to determine nonspecific binding. Data were collected on freshly stained cells using a Beckman-Coulter FC500 flow cytometer. Data analysis was done using Winlist software (Verity Software House, Topsham, ME).

**Analysis of Ki-ras mutations and quantitative PCR for mRNA expression levels of Rb, p16ink4a, cyclin D1, and p19/ARF.** Allele-specific oligonucleotide hybridization was done for Ki-ras mutation analysis. For Ki-ras analysis of lymphomas, DNA was purified from tumor tissue using the Promega Wizard Genomic DNA Purification Kit according to the instructions of the manufacturer (Promega Corp., Madison, WI). For lung adenomas, DNA was prepared for Ki-ras analysis from paraffin-embedded tumor samples by lysis in 100 μL digestion buffer [50 mmol/L Tris-HCl (pH 8.0), 0.5% Tween 20 with freshly added protease K at a final concentration of 200 μg/mL], Samples were digested overnight at 37°C and proteasine K inactivated the next day by heating at 95°C for 5 minutes. Two microliters (lymphoma) or 10 μL (lung adenoma) of DNA isolated from each tumor were amplified using specific primers for exons 1 and 2 of the Ki-ras gene. All reactions were carried out in a 100-μL reaction volume using either two units of AmpliTaq Gold (Perkin-Elmer Life Sciences, Boston, MA) or 4 units of Eppendorf Taq (Brinkman Instruments, Inc., Westbury, NY) with supplied reaction buffers [supplemental Mg2+ as either MgCl2 (2.5 mmol/L, Ampli Taq) or MgOAc (1 mmol/L, Eppendorf Taq)], 200 μmol/L deoxynucleotide triphosphates (dNTP), and amplifiers for exons 1 or 2 (exon 1 only for adenomas) of the Ki-ras gene, added at a final concentration of 0.2 μmol/L and overlaid with 100 μL mineral oil. After denaturation for 2 minutes at 94°C, samples were amplified by 40 cycles of denaturation for 1 minute at 94°C, annealing for 2 minutes at 55°C (lymphomas, exon 1) or 60°C (adenomas, exon 1), and extension for 2 minutes at 72°C, followed by a final extension step for 7 minutes at 72°C. Primers for exons 1 and 2 of Ki-ras were synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences for exon 1 (lymphomas) were forward 5’-ATGACTGAGTATAAATCTGTG and reverse 5’-TCGTACTCATCCACAAGAGT, which produced a 98-bp fragment; or for lung adenomas, forward 5’-GACTGATTAACTGTGTTG and reverse 5’-CAAAAGGCGGATGCACTTGT, which produced either a 383-bp fragment in mice harboring the Ki-ras-7 allele in B6 mice or a 345-bp fragment in mice with the Ki-ras-4 allele; primer

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sequences for exon 2 (lymphomas only) were forward 5′-TACAGGAAAAACATGATGTTAGGAGAA and reverse 5′-ATAATGCTGTAATATCTTCATATCAT-5′, which produced a 171-bp fragment. Each amplification reaction included procedure controls, which lacked tumor tissue but mock extracted and taken through the entire protocol, and negative buffer controls for PCR amplification reactions. All samples were amplified in a BioRad iCycler thermocycler. The sizes of PCR products were confirmed on a 2% agarose gel.

For lymphomas and lung adenomas homozygous for Ki-ras, 30 μL of PCR products, diluted in 170 μL of sterile water, were heat denatured and blotted onto a Nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a Schleicher & Schuell minifold II blot apparatus. For lung adenomas heterozygous for Ki-ras, Southern blotting was done to separate the two alleles for determination of the allele containing mutations. Twenty microliters of PCR products were electroeluted on 1% agarose gels, denatured for 30 minutes in 1.5 mol/L NaCl, 0.5 mmol/L NaOH, followed by neutralization for 30 minutes in 1 mol/L Tris-HCl (pH 7.4), 1.5 mol/L NaCl. Gels were subjected to capillary transfer overnight in 20 ml/C2 SSC onto Ambion BrightStar-Plus positively charged nylon membranes (Ambion, Austin, TX). The amplified DNA products were fixed to the membrane by UV cross-linking (Optional: for Ethidium bromide staining, membranes can be washed in 100 ml/C2 TAE to remove DNA). Neutralization for 30 minutes in 1 mol/L Tris-HCl (pH 7.4), 1.5 mol/L NaCl. Denhardt’s solution, 0.5% SDS, 100 μg/mL of salmon sperm DNA at 37°C for 1.5 hours. Membranes were hybridized overnight in prehybridization buffer containing 5 × 106 cpm/mL of a 20-bp oligonucleotide to mouse Ki-ras codons 12, 13 (lung adenomas, 12 and 13 only), or 61 (Clontech, Palo Alto, CA). The oligomers were 5′-end-labeled with 32P using T4 DNA polynucleotide kinase. After hybridization, filters were washed under stringent conditions (3 × 106 cpm/mL of a 20-bp oligonucleotide to mouse Ki-ras codons 12, 13 (lung adenomas, 12 and 13 only), or 61 (Clontech, Palo Alto, CA)).

<table>
<thead>
<tr>
<th>Gene (accession no.)</th>
<th>Primer sequence (F)</th>
<th>Primer sequence (R)</th>
<th>Real-time annealing temperature (°C)</th>
<th>PCR length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin (X03765)†</td>
<td>ATTGTGCTACAGGTGACAGAA</td>
<td>CAGGAGGAGCATTGTCTTGA</td>
<td>58.7</td>
<td>78</td>
</tr>
<tr>
<td>Rb (E12560; standard)</td>
<td>CTCCTGCACTTCTGTGAGTTTTCA</td>
<td>GAGAGGGCAGGCTTCAAGTTTGGT</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Rb (real time)</td>
<td>CTCCTGCACTTCTGTGAGTTTTCA</td>
<td>GAGAGGGCAGGCTTCAAGTTTGGT</td>
<td>59</td>
<td>63</td>
</tr>
<tr>
<td>Cyclin D1 (BC044841; standard)</td>
<td>TTGGTTGACCTGTCACCATATGGA</td>
<td>CAGGGTACCTGTCACCATATGGA</td>
<td>59</td>
<td>136</td>
</tr>
<tr>
<td>Cyclin D1 (real time)</td>
<td>TTGGTTGACCTGTCACCATATGGA</td>
<td>CAGGGTACCTGTCACCATATGGA</td>
<td>59</td>
<td>128</td>
</tr>
<tr>
<td>p16INK4a (AF332190; standard)</td>
<td>CAACGCCCGCAACCTCTTT</td>
<td>TTGAGGCAAGAGGCTACGTCATG</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>p16INK4a (real time)</td>
<td>CAACGCCCGCAACCTCTTT</td>
<td>TTGAGGCAAGAGGCTACGTCATG</td>
<td>59</td>
<td>77</td>
</tr>
<tr>
<td>p19/ARF (L67609)</td>
<td>GGCTAGAAGGAGTGTCAGGAAGGG</td>
<td>GCCATACATCATACATTCTGTCCAGG</td>
<td>61.2</td>
<td>63</td>
</tr>
</tbody>
</table>

*Additional exon-intron boundary information obtained from Ensembl Mouse Genome Browser.
†Same primer set used for both generation of the standard and real-time analysis.
Table 2. Effect of treatment and genotype of dam on litter size, genotype, gender ratio, and survival of offspring

<table>
<thead>
<tr>
<th>Treatment and genotype of dam/no. offspring</th>
<th>Litter size*</th>
<th>Genotype ratio (AHR&lt;sup&gt;−/−&lt;/sup&gt;/AHR&lt;sup&gt;+/+&lt;/sup&gt;)</th>
<th>Gender ratio (male/female)</th>
<th>Mortality at 10 mo (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-AHR responsive/n = 55</td>
<td>7.8 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.50 (32:21)</td>
<td>1.20 (30:25)</td>
<td>5.5 (3 of 55)</td>
</tr>
<tr>
<td>DBP-AHR responsive/n = 121</td>
<td>7.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.35 (65:48)</td>
<td>1.09 (63:58)</td>
<td>68.6 (83 of 121)</td>
</tr>
<tr>
<td>Control-AHR nonresponsive/n = 53</td>
<td>4.8 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.08 (27:25)</td>
<td>1.30 (30:23)</td>
<td>11.3 (6 of 53)</td>
</tr>
<tr>
<td>DBP-AHR nonresponsive/n = 102</td>
<td>4.1 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.71 (39:55)</td>
<td>0.82 (46:56)</td>
<td>89.2 (91 of 102)</td>
</tr>
</tbody>
</table>

<sup>*Litter size data are presented as mean ± SE (n ≥ 6); different superscripts indicate statistically significant difference (P < 0.05, Wilcoxon rank test). <sup>1</sup>Survival was lower in DBP-treated groups compared with controls for AHR-responsive dams and AHR-nonresponsive dams (P < 0.0001 for both cases).

Alleles. A 10-µL PCR reaction contained 1× GeneAmp buffer and 0.25 unit of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), 3.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L of each dNTP, 0.2 of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), with the Wilcoxon rank-sum test (in the SAS Npar1way procedure). or unequal variance (in the SAS Ttest procedure). Litter sizes were analyzed

**Statistical analysis.** For comparing diet treatments, the experimental unit is the pregnant female and the litters represent clusters for analyzing mRNA expression levels from real-time RT-PCR. A mixed model used for survival modeling with litters as clusters (marginal model) to get measurements made on offspring. Cox proportional hazard regression was concluded the reaction. PCR products were separated on Novex 8% Tris-borate EDTA gels (Invitrogen Life Technologies, Carlsbad, CA). AHR<sup>−/−</sup> homozygotes yielded two PCR products of 158 and 148 bp, respectively. AHR<sup>+/+</sup> heterozygotes yielded two PCR products of 158 and 148 bp, respectively. AHR<sup>+</sup> homozygotes yielded a single product of 148 bp. The molecular weight ladder wasMspI-cut pBR322 DNA (New England Biolabs, Beverly, MA). Ethidium bromide was used to stain the DNA; visualization was by UV.

**Results**

The treatment protocol used here resulted in four experimental groups of offspring (Table 2). Treatment of pregnant mice with a single 15 mg/kg dose of DBP by gavage on day 17 of gestation did not elicit acute maternal or fetal toxicities; there was no significant DBP-dependent reduction in litter size (Table 2) or birth weight (data not shown). The AHR-responsive mothers had significantly larger litters and the ratio of responsive to nonresponsive pups was greater than unity (Table 2), as has been previously observed with the B6D2F1 compared with D2 dams in the 3-methylcholanthrene transplacental carcinogenesis model (45). Beginning at ~3 months of age, offspring exposed to DBP in utero had difficulty in breathing, as well as anemia and hypoxia, that resulted in morbidity requiring euthanasia. Most mortality occurred at 3 to 6 months of age (Fig. 1). Gross necropsy revealed large thoracic masses and enlarged spleens, livers, and lymph nodes. There was no sex difference with respect to DBP-dependent mortality (data not shown). The cause of death was determined to be an aggressive lymphoma involving numerous organs. Lymphoblasts were arranged in sheets, had medium to large nuclei with one to multiple nucleoli, and had a high number of mitotic figures. Tumor cell degeneration and death was frequent and resulted in a starry sky appearance (data not shown). Immunohistochemistry showed that these cells were CD3+. Flow cytometric analysis of the thymic tumors from four mice confirmed a T-cell phenotype (CD3+, B220−). Of three tumors that were further characterized, two expressed CD4−CD8− predominately and one expressed both CD4−CD8+ and CD4+CD8+ phenotypes. Based on these findings, the lymphomas were classified as a T-cell lymphoblastic lymphoma. An uncommon heart lesion was noted in a few of the offspring exposed to DBP in utero and was characterized by an endothelial proliferation resembling hemangiosarcoma.

When the study was terminated at 10 months, 89% and 69% of the mice born to DBP-treated nonresponsive and responsive mothers, respectively, had succumbed to lymphoma. Few mortalities were observed over this same period in offspring born to mothers given corn oil alone and none of these mice had neoplasms. Looking at all offspring together, a nonresponsive maternal AHR phenotype enhanced risk of mortality by 1.8-fold compared with the responsive maternal AHR phenotype (Fig. 1A; P = 0.004). When the phenotype of the offspring is considered, offspring with responsive AHR phenotypes had greater risk of mortality in both maternal phenotypes (Fig. 1B). When the mother was AHR responsive, the estimated increased risk for responsive offspring was 2.1-fold (P = 0.007); when the mother was AHR nonresponse, the estimated increased risk was only ~1.4-fold (P = 0.21). Although the observed effect of offspring phenotype was larger in AHR-responsive mothers, the difference was not statistically significant (interaction in two-way factorial model, P = 0.3). The ratio of AHR-responsive/AHR-nonresponsive offspring was somewhat lower (0.71) in litters from nonresponsive mothers treated with DBP (Table 2). This may indicate in utero toxicity in responsive pups exposed to a higher dose of DBP due to greater carcinogen bioavailability from the nonresponsive mothers, in agreement with results previously obtained with other PAHs (19, 20, 46).

All mice surviving to 10 months of age that were exposed in utero to DBP exhibited hyperplasia, adenoma, adenoma with progression, and/or carcinoma of the lung compared with a 7% to 10% spontaneous incidence in offspring born to mothers given vehicle alone (Table 3). The average number of lung tumors in tumor-bearing mice exposed to DBP was 13.5 to 14.0, compared with 1.2 to 1.3 in controls (Table 3). No significant effect of sex or genotype was discerned on lung tumor multiplicity (data not shown). Liver tumors (primarily foci with the remainder adenomas) were also observed in mice surviving to 10 months but only if exposed to DBP in utero. For liver, there was a marked sex difference in tumor incidence (Table 3), such that males had a much greater incidence
than females. There was no effect of phenotype on liver tumor incidence in offspring born to AHR-responsive mothers (Table 3). In offspring born to AHR-nonresponsive mothers, there seemed to be an effect of AHR phenotype but only a few mice (n = 11) survived to 10 months of age.

Thymic lymphomas from morbid mice euthanized between 3 and 6 months of age and lung tumors from mice necropsied at 10 months of age were analyzed for mutations in Ki-ras, TP53, and β-catenin. No mutations in Ki-ras (exon 1, codons 12 and 13; exon 2, codon 61) or TP53 (exons 5-8) were observed in thymic lymphomas. In lung, four tumors spontaneously formed in control mice were determined to be negative for Ki-ras mutations in codons 12 and 13. Only 3 of 14 (21%) lung tumors from mice exposed to DBP in utero harbored Ki-ras mutations [1 G→T transversion in codon 12 (Cys), 1 G→A transition in codon 12 (Asp), and 1 G→C transversion in codon 13 (Arg)]. This incidence is much lower than previously observed (80%) in offspring from B6D2F1/D2/C2 crosses exposed to 3-methylcholanthrene in utero (46–48). We also examined the expression of Rb, p16Ink4a, cyclin D1, and p19/ARF in lung adenomas by real-time RT-PCR. mRNA levels for p16Ink4a and cyclin D1 were unchanged, Rb was decreased by 50%, and 1 G→C transversion in codon 13 (Arg)]. This incidence is much lower than previously observed (80%) in offspring from B6D2F1/D2/C2 crosses exposed to 3-methylcholanthrene in utero (46–48). We also examined the expression of Rb, p16Ink4a, cyclin D1, and p19/ARF in lung adenomas by real-time RT-PCR. mRNA levels for p16Ink4a and cyclin D1 were unchanged, Rb was decreased by 50%, and p19/ARF was enhanced 35-fold (Table 4).

Discussion

A number of studies in animal models have shown that environmental carcinogens are capable of inducing tumors transplacentally (8, 10). Epidemiologic studies indicate that in utero exposure to xenobiotics can render children at increased risk for a number of diseases, including cancer (1–7). For example, exposure to the antiseizure drug phenytoin in utero has been linked to development of T-cell lymphoblastoid lymphoma in humans (49).

Treatment of the pregnant, AHR-responsive, B6D2F1 (crossed to an AHR-nonresponsive D2 male) or D2 (crossed to a B6D2F1 male) mouse with 3-methylcholanthrene produces lung adenomas in offspring by 1 year of age (10–14, 23, 45–48). The transplacental tumor response in this model is a function of the AHR phenotype. A nonresponsive AHR phenotype (D2, AHR<sup>d/d</sup>) in the mother markedly enhances lung tumor incidence regardless of the fetal/offspring phenotype. A responsive fetal/offspring phenotype markedly enhances lung tumor incidence irrespective of maternal phenotype. Thus, the highest tumor incidence is observed in AHR-responsive offspring born to AHR-nonresponsive mothers, presumably caused by fetal metabolic activation of greater amounts of 3-methylcholanthrene due to low clearance/metabolism by the mother. In the present study, we used the potent PAH carcinogen DBP, which, unlike 3-methylcholanthrene, is present in the environment. In addition, we used a B6129F1 × 129 cross (the 129 strain has the same AHR<sup>d/d</sup> genotype as the D2 mouse) rather than the previously used B6D2F1 × D2 cross. The advantage of using the B6129F1 was that this is the more common genetic background for knockout mouse models that could be employed in future studies to examine the mechanism(s) of PAH-dependent transplacental carcinogenesis.

Rather than development of lung adenomas at 1 year, exposure of pregnant mice to DBP led to appearance of very aggressive lymphomas in offspring beginning at 3 months of age that were determined by histopathology and immunohistochemistry to be a
T-cell lymphoblastic lymphoma. In a previous study (29), DBP treatment (daily, five times a week for 3 weeks at a dose of 1.07 mg/kg; total dose of ~15 mg/kg body weight) of mice on the mixed B6129 genetic background resulted in tumors after 12 months in a number of tissues, including ovary, skin, lymphoma, uterus, and liver. The lymphomas were classified as lymphoblastic (primarily of T-cell origin) or follicular (large B-cell) lymphomas. In this same study, the cancer incidence at most sites markedly declined in Cyp1b1 knockout mice of the same genetic background. These results are consistent with other studies showing Cyp1b1 to be the most efficient cytochrome P450 enzyme in bioactivation of DBP (15–18, 29) and to be required for DBP- or 7,12-dimethylbenz(a)anthracene–induced carcinogenesis (29, 34–36, 50).

As in the B6D2F1 × D2 crosses in which the dam was treated with 3-methylcholanthrene, a nonresponsive AHR maternal phenotype and a responsive fetal phenotype enhanced cancer risk in offspring, but in our study the primary cancer resulted in early mortalities from an aggressive lymphoma rather than development of lung tumors at 1 year. The lymphomas were diagnosed as a T-cell lymphoblastic lymphoma and observed only in offspring from mothers exposed to DBP during pregnancy. The study design did not involve cross-fostering of pups so that DBP exposure could have continued throughout lactation from deposition of DBP in mother’s milk. Subsequent studies would be required, employing a cross-fostering design, to determine how much of the carcinogenicity of DBP was due strictly from transplacental exposure and how much from nursing. Previous studies with transplacental exposure to 3-methylcholanthrene that did (12) or did not (48) employ foster mothers would indicate that little of the 3-methylcholanthrene transplacental carcinogenesis comes from lactation exposure. In either case, the present experimental design would better recapitulate possible human exposures.

All mice surviving to 10 months of age, exposed to DBP in utero, exhibited multiple lung tumors (i.e., hyperplasia, adenoma, adenoma with progression, and/or carcinoma). The spontaneous incidence of lung tumors in the B6129F1/C2129 cross was 7% to 10%, markedly higher than the relatively resistant B6D2F1/C2D2 pups (21). Liver tumors in offspring were also caused by DBP exposure to the pregnant mouse, but as has been seen in other mouse models, including the transplacental 3-methylcholanthrene model, liver tumor incidence was much higher in males.

### Table 3. Lung and liver tumors in offspring surviving to 10 months of age

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Multiplicity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-AHR–responsive dam/α = 50</td>
<td>5 of 50 (10%)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>DBP-AHR–responsive dam/α = 38</td>
<td>38 of 38 (100%)</td>
<td>14.0 ± 1.5</td>
</tr>
<tr>
<td>Control-AHR–nonresponsive dam/α = 46</td>
<td>3 of 46 (7%)</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>DBP-AHR–nonresponsive dam/α = 11</td>
<td>11 of 11 (100%)</td>
<td>13.5 ± 1.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Multiplicity</th>
<th>Offspring phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Responsive</td>
</tr>
<tr>
<td>DBP-AHR–responsive dam/α = 38</td>
<td>14 of 20 (70%)</td>
<td>1 of 18 (6%)</td>
<td>5 of 14 (36%)</td>
</tr>
<tr>
<td>DBP-AHR–nonresponsive dam/α = 11</td>
<td>4 of 6 (67%)</td>
<td>0 of 5 (0%)</td>
<td>0 of 3 (0%)</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SE for multiplicity.

### Table 4. Quantitative real-time PCR of Rb, p16, cyclin D1, and p19 in normal lung tissue from pups born to untreated mothers and in lung adenomas from pups born to mothers exposed to DBP

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Rb*</th>
<th>p16*</th>
<th>Cyclin D1*</th>
<th>p19*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>9.0 ± 1.3</td>
<td>3.6 ± 0.8</td>
<td>24.9 ± 2.8</td>
<td>0.3 ± 0.03</td>
</tr>
<tr>
<td>DBP-lung adenomas (n = 13)</td>
<td>4.4 ± 0.6</td>
<td>3.5 ± 0.4</td>
<td>27.8 ± 4.0</td>
<td>10.6 ± 3.9</td>
</tr>
<tr>
<td>P values for comparison of the two sets</td>
<td>P = 0.041</td>
<td>P = 0.702</td>
<td>P = 0.9727</td>
<td>P &lt; 0.00011</td>
</tr>
</tbody>
</table>

*All values are normalized to β-actin expression (×1,000) and presented as mean ± SE.
1From mixed model with random litter effects.
2From standard, equal-variance t test (no evidence of litter effects).
3From unequal-variance t test on log scale (no evidence of litter effects).
None of the thymic lymphomas analyzed by single-strand conformation polymorphism exhibited any mutations in Ki-ras, β-catenin, or TP53. The p53-null mouse on the B6/129 genetic background exhibits mortality from a T-cell lymphoma with a time course similar to DBP in the present study (51). We also did not observe any TP53 mutations in lung tumors in mice surviving to 10 months of age, consistent with the 3-methylcholanthrene, B6D2F1 × D2 transplacental model (52). TP53 mutations are frequently observed in human lung cancers (53) whereas the role of TP53 mutations in T-cell lymphomas is not entirely clear, perhaps owing to the number of different cell lineages from which the lymphomas arise. Alterations in p53 are frequently found in human leukemia-lymphoma cell lines (54) and certain T-cell lymphomas in humans have a high incidence of TP53 mutations (55) whereas others display p53 dysregulation without mutation (56). In this study, 3 of 14 lung adenomas from offspring born to mothers exposed to DBP during pregnancy exhibited Ki-ras mutations, a frequency markedly lower than seen following transplacental exposure to other PAHs in other mouse strains (47). Real-time PCR measurements of p16 and cyclin D1 expression in lung adenomas did not show any change in expression levels compared with normal lung tissue; however, Rb levels were reduced by 50% and p19/ARF levels elevated 35-fold. A reduction in Rb protein expression in lung adenomas has previously been observed in the 3-methylcholanthrene transplacental tumor model of Rollins et al. (57). Rb normally forms a repressor complex with E2F1 which down-regulates p19/ARF expression. Hence, the increase in p19/ARF may be due to the significant reduction of Rb available to form the repressor complex.

In conclusion, we have shown for the first time that transplacental (and possibly lactational) exposure to a PAH can result in a high incidence of mortality in young adult offspring due to an aggressive T-cell lymphoblastic lymphoma, a disease also observed in humans.

The AHR phenotype is important in response to this environmental carcinogen; a nonresponsive maternal phenotype enhanced risk, presumably due to greater bioavailability to the fetus, and risk was further augmented by a responsive fetal phenotype, presumably due to induction of Cyp1a1 and/or Cyp1b1 bioactivation in fetal targets. The nonresponsive phenotype of the 129 mouse is due to the d allele, which shifts the dose-response of chemicals, for which toxicity is mediated through AHR by an order of magnitude compared with a responsive allele (58). Studies with a humanized AHR mouse indicate that human AHR resembles mouse AHR d allele with respect to ligand binding affinity and response (59). The human AHR also exhibits genetic polymorphisms but it is still not clear what effect the various phenotypes have on expression of AHR-regulated genes and toxicity of AHR ligands (60). A recent study found that a polymorphism in exon 10 at codon 554 was significantly associated with reduction in survival of patients with soft tissue sarcomas (61).

Mice surviving to 10 months of age had a 100% incidence of multiple lung adenomas; a significant percentage of survivors, mostly males, also developed liver tumors. Exposure of fetal and neonatal humans to carcinogens such as DBP (and other PAHs) may contribute to the enhanced susceptibility of children and young adults to lymphomas and leukemias, as well as other cancers, later in life.

Acknowledgments

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