Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse.

Barbara D. Abbott, Gary Held, Janet Diliberto, and Judith Schmid, National Health and Environmental Effects Research Laboratory, USEPA, Research Triangle Park, NC 27711 USA

Abstract

The aryl hydrocarbon receptor (AhR) is a transcriptional regulatory protein which binds to upstream DNA response elements of target genes. Activation of the AhR by binding of ligands such as dioxins, furans, and PCBs can induce immune deficiencies, embryo/fetotoxicity and reproductive toxicity. Investigations of the diverse biological responses mediated by the AhR led to production of a transgenic mouse in which the gene coding for the Ah receptor was inactivated. AhR-deficient mice were fertile and at maturity exhibited immune system impairment and hepatic fibrosis. Our laboratory received several of these homozygous knockout (-/-) mice and mated them with wild type (+/+) C57BL/6N to generate large numbers of heterozygotes (+/-). The 5 -/- males in our colony were mated with a total of 45 heterozygous (+/-) females. Offspring of these matings were genotyped and 38 -/- pairs were mated. Observations included age at cohabitation, time from cohabitation to birth of litter, pregnancy rate, maternal survival during pregnancy/lactation, number live and dead pups at birth, pup survival (lactation and post-weaning), sex ratio of pups, genotype distribution and differential survival by genotype and sex. Litter sizes of -/- dams were reduced compared to +/- and mortality was increased among lactating -/- dams (35%) compared to the +/- dams (0%). More litters of -/- dams had pup deaths during lactation and mortality of -/- pups (both sexes) increased after weaning compared to +/- pups. Across all litters the sex ratio and genotype distribution appeared as expected. Reproductive success was adversely affected by the knockout of the AhR gene with decreased survival of conceptuses, lactating dams, and weaned pups. Additional study is needed to reveal the etiology of these effects.

Introduction

The aryl hydrocarbon receptor (AhR) is a transcriptional regulatory protein which binds to upstream DNA response elements of target genes[1-3]. This receptor is a member of the Per-Arnt-Sim family of transcriptional regulators which have a basic helix-loop-helix component in the DNA-binding regions. Ligands for this receptor include environmental contaminants in the polyhalogenated aromatic hydrocarbon family, including dioxins, furans, and coplanar biphenyls. Activation of the AhR by these compounds is associated with disruption of almost every hormone system which has been examined and responses to activation include developmental and reproductive toxicity. AhR is expressed in a specific spatial and temporal pattern in the developing embryo/fetus[4]. Prenatal exposure to dioxin can induce teratogenesis, immune deficiencies, embryo/fetotoxicity and reproductive effects including delayed puberty, decreased

sperm count, altered reproductive behavior, structural reproductive tract abnormalities, and reduced fertility. Investigations of the diverse biological responses mediated by the AhR have led to production of a transgenic knockout mouse in which homologous recombination methods were used to inactivate the gene coding for the Ah receptor[5,6]. The -/- mice exhibited immune system impairment and hepatic fibrosis. However, the -/- mice were unaffected by exposure to high levels of TCDD that induced pathological responses in wild type littermates[7]. Initial reports indicated that knockout mice were fertile, but had reduced litter size and survival of neonates was reduced during the first week after birth. Our laboratory received several of these mice and in the process of expanding the colony, we accumulated reproductive data on large numbers of these animals.

Methods

The AhR-deficient (AhR-/-) mouse line was obtained from P. Fernandez-Salguero and F. J. Gonzalez, at the National Cancer Institute of NIH. This knockout transgenic was prepared by homologuous recombination in embryonic stem cells targeting the basic helix-loop-helix domain of the AhR gene, as described in Fernandez-Salguero et al.[1]. The 6 female and 3 male -/- mice which we received (designated F0) were mated with C57BL/6N wild type mice (Charles River Laboratories, Raleigh, NC) to produce +/- genotypes (F1). Some of the F0 male and female mice were mated to give additional -/- offspring (F1). At ages ranging from 53 to 84 days the F1 female +/- mice were mated to -/- males (F0 and F1). Each male was housed continuously with up to 3 females and mated (as determined by presence of copulatory plug) females were removed and replaced with nonpregnant females until all F1 females were pregnant. This mating scheme was necessary due to the limited number of -/- males in the colony. Results of these matings are listed as group (1) in the tables. The F2 offspring, +/- and -/-, were genotyped by PCR analysis and at 8-10 weeks of age pairs were mated as -/- female to -/- male, -/- female to +/- male, or +/- female to -/- male (listed as groups 2, 3, and 4 in the tables, respectively). All of these matings were at a 1:1 ratio of male:female (unlike the previous continuous mating scheme) and lineage records of all mice (F0 and F1) were used to avoid consanguineous matings. Females which did not become pregnant within 2 weeks were removed and housed for an additional 2 weeks with a proven male (one which had sucessfully impregnated a female). The F3 offspring of all F2 matings were observed to postnatal day (PND) 45 for survival. Data collected included age at cohabitation of males and females; pregnancy status; time from cohabitation to birth of litter; deaths of dams during pregnancy or or lactation; mean number of implantations in the uteri of the F2 females (not determined for F1 dams); number of live and dead pups at birth; deaths during lactation; age of pups at death; age, sex and genotype at weaning. Genotype was determined for pups that died during lactation when possible. Statistical analysis by ANOVA, chi-square test, and Fisher's exact test were used to assess effect of genotype on time from cohabitation to birth of litter, survival of dams and pups, litter size, time of pup death, and distribution of genotypes and sex ratio.

Results and Discussion

The AhR transgenic mice heterozygous for the knockout (+/-) were previously reported to be fertile with a normal distribution of genotypes among offspring of +/- x +/- matings[5]. However 40 to 50% of -/- AhR offspring were dying within 1 to 4 days after birth. In -/- x -/- matings the litter sizes were reported to be 1-4 pups[2]. Transgenic mice from that colony were shipped to our facility where matings were undertaken to expand the colony. In the process of

increasing the -/- population the reproductive success and survival of the +/- and -/- animals were assessed. This study found a deleterious effect of AhR deficiency on survival of the lactating female, on litter size, on post-implantation loss, on numbers of litters with deaths during lactation, on mean time from cohabitation to birth, and on survival after weaning of the pup.

The -/- males (F0, F1) mated to multiple females were fertile and impregnated females within a relatively short time. The time from cohabitation to birth of a litter averaged 21.7 ± 0.3 days for these matings. The F2 males (+/-, -/-) in 1:1 matings also produced litters in 80.6% of the matings and the mean time from cohabitation to birth was 23.5 ± 0.7 days. This was a significant increase in time to birth among -/- x -/- matings and this could be a result of increased time to sucessful mating (attributable to either male or female) or increased pregnancy duration. A significant increase in deaths of -/- females occurred during the lactational period, with only 1 death among the +/- dams. The number of implantations was similar between +/- and -/- females and was within the range expected for mice of this genetic background. However the total number of pups at birth was significantly decreased in -/- compared to +/- litters. Although the mean day of death for pups dying during the lactational period is similar to that reported previously, few pups died in any of the litters. However, the number of litters with a lactational death was significantly increased for -/- litters.

Most of the postnatal -/- deaths occurred in the first week after weaning. Pups were weaned after 3 weeks and significantly more -/- pups of both sexes died compared to +/- pups (F1 -/- 26.4%, +/- 6.9%, sexes combined). Analysis indicated that postnatal deaths were evenly distributed across the sexes. Chi-square analysis also demonstrated a normal distribution of sexes and genotypes among the offspring. The survival from birth to PND 45 for +/- pups was 70±6.0% and 70%±10 (male and female, respectively), significantly fewer -/- pups (52 % ±3.3 of -/- females and 53%±4.5 of -/- males) survived this period.

Although the females are fertile, these data raise issues concerning the ability of -/- females to maintain conceptuses during pregnancy and survive the physiological stresses of lactation. The -/- pups also have difficulties surviving postweaning adaptations. The normal distribution of genotypes and sexes at weaning suggests that AhR-deficiency is not differentially affecting survival of male vs female embryos or pups. If the number of implantations is considered 100%, then the number of individuals in the next generation decreases to 67% by birth, 30% by weaning, and 26% by PND45. These deaths can be mainly attributed to prenatal resorption (~33%), deaths of nursing dams (~35%), and deaths of weaned pups (~26%) . The inactivation of the AhR gene leads to adverse reproductive performance with substantial attrition in the next generation.

Acknowledgments The AhR-deficient transgenic mice were generously provided for this study by Dr. Pedro Fernandez-Salguero and Dr. Frank J. Gonzales of the National Cancer Institute, NIH, Bethesda, MD. Dr. Linda S. Birnbaum played a key role in obtaining the transgenic mice and as an expert in this field of research, her recommendations and critical review of the abstract were valued highly. The expert assistance of C. Wood and J. Brown were invaluable in the completion of these studies.

Literature Cited

(1) Birnbaum, L.S. *Receptor-Mediated Biological Processes: Implications for Evaluating*

Carcinogens. Progress in Clinical and Biological Research Vol 387. **1994,** Wiley-Liss; NY; p139-154.

(2) Whitlock, J.P. *Annu Rev Pharmacol Toxicol* **1990**,30,251-277.

(3) Poland, A.; Knutson, J.C. *Annu Rev Pharmacol Toxicol* **1982**,22,517-554.

(4) Abbott, B.D.; Birnbaum, L.S.; Perdew, G.H. *Developmental Dynamics***1995**,204,133-143.

(5) Fernandez-Salguero, P.; Pineau, T.; Hilbert, D.M.; McPhail, T.; Lee, S.S.T.; Kimura,S; Nebert, D.W.; Rudikoff, S.; Ward,J.M.; Gonzales, F.J. *Science* **1995**,268,722-726.

(6) Gonzalez,F.J.; Fernandez-Salguero, P.; Lee, S.S.T.; Pineau, T.; Ward,J.M *Toxicology Letters***1995**,82/83,117-121.

(7) Fernandez-Salguero, P.; Hilbert, D.M.; Rudikoff, S.; Ward,J.M.; Gonzales, F.J. *Toxicol. Appl. Pharmacol.***1996**,140,173-179.

(This is an abstract of a proposed presentation and does not necessarily reflect EPA policy).

BREEDING DATA							
Group	Generation/ Genotype Male	Female	# Females	% Pregnant	Mean days cohabitation to birth	% Dead in pregnancy (# females)	% Dead in lactation (# females)
1	F0/F1 -/-	F1 +/-	45	73%	21.7 ± 0.3	0% (0)	0% (0)
2	F2 -/-	F2 -/-	38	90%	23.5 ± 0.7*	3% (1)	35%*** (12)
3	F2 +/-	F2 -/-	9	89%	22.6 ± 1.1	11% (1)	13% (1)
4	F2 -/-	F2 +/-	9	100%	24.9 ± 1.8	0% (0)	11% (1)
Group 1 vs 2: *P<0.05; ***P<0.001							

LITTER DATA										
		# Litters	Mean Implant-ations	Mean live/litter	Mean dead/litter at birth	Mean total pups/litter	Mean deaths/ litter in lactation	Mean PND of death	% (#) litters with dead at birth	% (#) litters w/ deaths in lactation
1	+/-	33	NA	8.3 ± 0.4	0	8.3 ± 0.4	0.4 ± 0.2	2.0 ± 1.0	0	18% (6)
2	-/-	34	8.96± 0.1	6.9 ± 0.4*	0.1 ± 0.1	7.0 ± 0.4*	0.6 ± 0.2*	5.0 ± 1.2*	9% (3)	29%* (10)
3	-/-	8	8.0± 0.3	5.4 ± 0.9#	0.8 ± 0.6	6.1 ± 0.5	0	NA	25% (2)	0
4	+/-	9	8.63± 0.2	8.0 ± 0.5	0	8.0 ± 0.5	0.3 ± 0.2	19.0 ± 0	0	22% (2)
Group 1 vs 2: *P<0.05; Group 1 & 2 vs 3: #P<0.05.										

WEANED PUP DATA & SURVIVAL TO PND 45							
	Total pups weaned	Mean age at wean^	Mean age at death^	+/- % dead (# weaned) Male	Female	-/- % dead (# weaned) Male	Female
1	247	23.2 ± 0.3	36.4 ± 3.2	9% (58)	6% (73)	35% (63)	15% (53)
2	106	22.4 ± 0.6	32.2 ± 1.8			6% (47)	17% (59)
3	34	22.7 ± 1.4	32.0 ± 5.1	0% (10)	17% (6)	10% (10)	38% (8)
4	42	21.3 ± 0.4	27.2 ± 3.2	0% (9)	0% (12)	13% (8)	15% (13)
^Across genotypes and sexes. +/- vs -/- : *P<0.01among females; P<0.001 among males.							