Are Developmentally-exposed C57BL/6 Mice Insensitive to Suppression of TDAR by PFOA?

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Abstract

Perfluorooctanoic acid (PFOA) is an environmentally persistent fluorinated compound that is present in biological samples worldwide and associated with multisystem toxicity in laboratory animal models. Several studies have reported suppression of T-cell-dependent antibody responses (TDAR) in adult rodent models after 15 or 28 days of exposure. A related compound, perfluorooctane sulfonate (PFOS), was reported to suppress TDAR in developmentally-exposed mice. The developmental effects of PFOA exposure on TDAR have not been explored; therefore, the objective of our study was to determine if TDAR suppression would occur in developmentally-exposed mice. Pregnant C57BL/6 mice were given 0, 0.5, or 1 mg PFOA/kg BW in drinking water from gestation day (GD) 6-17. At postnatal day (PND)2, litters/dam were reduced to three males and three females. On PND21, female offspring were weaned and separated and on PND43, were intravenously immunized with sheep red blood cells. Serum for evaluation of IgM titers and PFOA concentrations was collected 5 d later. Booster immunizations were given 14 d later; serum for evaluation of IgG titers and PFOA concentrations was collected 5 d after. Litter weights were statistically decreased by 10% in the 1 mg/kg group relative to controls, but liver weights, lymphoid organ weights, and TDAR did not differ in female offspring by dose. Mean PFOA serum concentrations were 122 ng/ml (0.5 mg/kg) and 183 ng/ml (1 mg/kg) and less than 1 ng/ml for controls. PFOA serum concentrations in offspring were 400-fold lower than serum concentrations reported to suppress TDAR in adults; however, mice exposed during development did not survive doses higher than 1 mg/kg. Therefore, although TDAR in adult mice is sensitive to PFOA exposure, the doses and exposure scenario of this study did not induce developmental immunotoxicity (DIT). C57BL/6 mice likely are more sensitive to the overt developmental toxicity of PFOA than to potential DIT.

Introduction

Perfluoroalkyl acids (PFAAs) are fluorinated compounds used to manufacture myriad consumer products, from adhesives and water/stain repellent surfaces to nonstick coatings and lubricants. Certain perfluorinated precursors undergo chemical, microbial, and photolytic degradation to a limited number of extremely stable degradation products, including perfluorooctanoic acid (PFOA). PFOA is also a polymerization aid used in the manufacture of fluorinated polymers and elastomers. As a result of its use and as a degradation product of other perfluorinated compounds, PFOA is widespread in environmental media and has been reported in the serum and tissues of humans and wildlife (Lau et al., 2007). Concerns about PFOA's toxicological effects prompted a draft risk assessment by the U.S. Environmental Protection Agency (EPA), which was completed in 2005. Currently, toxicity data for human health risk assessment is still being generated.

The studies cited in the preliminary risk assessment reported reductions in lymphoid organ weights and suppression of *de novo* antibody synthesis in adult rodents (Yang et al., 2000, 2001, 2002). In these initial and subsequent studies with adult rodents (DeWitt et al., 2008; Loveless et al., 2008), 10-28 days of oral exposure to up to 30 mg PFOA/kg body weight (BW) reduced antigen-specific IgM antibody synthesis. We previously reported that the lowest observed adverse effect level (LOAEL) for alteration of primary (IgM) antibody titers in adult mice was 3.75 mg/kg (7.4×10^4 ng/ml in serum), which is approximately 150 times greater than concentrations in highly exposed adult human populations (DeWitt et al., 2008). However, it is generally accepted that the developing immune system is more susceptible to perturbations than the adult immune system. To date, there is only one published account of developmental immunotoxicity (DIT) in a rodent model exposed to PFAAs. Keil et al. (2008) reported that

perfluorooctane sulfonate (PFOS) induced DIT in $B_6C_3F_1$ male mice exposed from gestational day one (GD1) through GD17 and that the IgM antibody response was suppressed in male offspring when assessed as adults. It is probable that PFOA may also have an effect on the developing immune system and protecting potentially susceptible subpopulations requires evaluating the immune response in animals developmentally exposed to PFOA. Therefore this study was designed to investigate the potential DIT of PFOA in a rodent model.

Materials and Methods

Animals

C57BL/6N timed-pregnant female mice (7-8-wk-of-age) were purchased from Charles River Laboratories (Raleigh, NC) and delivered to the East Carolina University (ECU) Brody School of Medicine (BSOM) animal facility (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care) at gestational day six (GD6). Once at the ECU's animal facilities, animals were singly housed in polycarbonate cages with corn cob bedding, soft bedding material, and a hiding tube. They were provided a 12-hr light:dark cycle (light, 0600-1800 hours; dark, 1800-0600 hours), maintained at 23 ± 3 ºC and 30-70% relative humidity, and given *ad libitum* access to both food (5P00 Prolab RMH 3000) and water. All procedures employed in this study involving animal models were approved in advance by the Institutional Animal Care and Use Committee of ECU.

Experimental design

Keil et al. (2008) exposed pregnant C57BL/6 dams to various doses of PFOS from GD1-GD17 and reported decreased IgM antibody synthesis in male offspring. However, in a pilot study using the same exposure paradigm, C57BL/6 dams receiving 0, 1, or 5 mg PFOA/kg from GD1-GD17 produced an insufficient number of litters for statistical analysis. A second pilot

study was performed with exposure beginning at GD6, one day prior to the beginning of fetal hematopoeisis in rodents. In this second pilot study, the 5 mg PFOA/kg dose decreased neonatal survival by 75% (6/8 litters died by PND6) and pups from only two litters survived until weaning. Consequently, for the current study, dams received 0.5 or 1 mg PFOA/kg in drinking water from GD6 through GD17. Dosing water was changed and water consumption (based on water bottle weights) was recorded twice weekly. Vehicle controls received deionized water for the same duration as treated animals. After the dosing period, all water bottles were changed to vehicle water. On postnatal day two (PND2), pups were weighed, counted, sexed, and culled to three males and three females per dam. Dams that did not deliver enough male or female pups received extra pups from dams within the same dose group and only dams that successfully delivered at least three pups were kept in the study. Pups were weighed weekly from PND7-20. On PND20, all male pups were euthanized and on PND21, 16 female pups per dose were sorted randomly into IgM (N = 8/dose) and IgG (N = 8/dose) endpoint groups. Littermates were included across IgM and IgG groups, but not within IgM or IgG groups.

Dosing solutions

PFOA was purchased from Sigma-Aldrich (St. Louis, MO) as its ammonium salt (≥ 98% purity, lot 1349401 14907133). PFOA drinking water dosing solutions were prepared based on total compound weight, as described by DeWitt et al. (2008) at concentrations of 6.7 or 3.35 mg/L (to provide doses of 1 or 0.5 mg/kg/day, respectively, based on average daily water consumption and animal body weights).

Organ weights

Immediately following euthanasia, the left adrenal gland, liver, spleen, and thymus were removed from each animal, weighed, and frozen at -80ºC for future histological analysis.

IgM and IgG antibody titers

Animals from all groups were immunized on PND44 (6 wk-of-age) by intravenous injection of 4.0 x 10^7 sheep red blood cells (SRBC) in 0.2 ml of sterile saline. Five days later, animals from the IgM groups were euthanized by carbon dioxide inhalation and exsanguinated by neck vein transection. Blood was collected and held at room temperature for 30 min, centrifuged at 4ºC to separate serum, and then frozen at -80ºC until analysis of IgM antibody titers. Two weeks after the primary immunization, animals from the IgG groups were given a booster immunization of SRBC at the same concentration as the initial immunization. Five days later, animals were euthanized by carbon dioxide inhalation and exsanguinated by neck vein transection. Blood was collected and treated as described for IgM groups.

IgM and IgG antibody titers were determined as described previously (DeWitt et al., 2005). Briefly, flat bottom 96-well Immunolon-2 ELISA microtiter plates (Dynatech Labs, Chantilly, VA) were coated with 125 µL of 2 µg/ml of SRBC membranes [1.46 mg/ml stock solution diluted in phosphate-buffered saline (PBS), prepared according to Temple et al. (1995)] and then incubated at 4°C for at least 16 hr. Each plate included 20 wells coated with pooled serum collected from healthy mice 5 days after primary immunization with SRBC, and 16 wells contained 100 µL PBS as blanks. After washing, blocking of nonspecific binding, and addition of serum samples (serially diluted from 1:8 to 1:4,096), secondary antibody (goat anti-mouse IgM or IgG horseradish peroxidase; Accurate Chemical and Scientific Corp., Westbury, NY) was added. Following three washes and addition of substrate [2,2´-azino-bis(3 ethylbenzthiazo-line-6-sulfonic acid diammonium salt (20 mg; Sigma-Aldrich) added to phosphate-citrate buffer with urea hydroxide peroxide (Sigma-Aldrich) in 100 ml of distilled water, 0.05 M final

solution], plates were incubated for 45 min at room temperature and then read at 410 nm on a BioTek Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT).

Serum PFOA concentrations

PFOA concentration was determined from aliquots of serum collected for measurement of IgM and IgG titers. Samples were prepared as described in Reiner et al. (2009). In brief, serum samples were thawed by placement in cool water and vortexed 30 seconds prior to sampling. An aliquot of serum (25 µl) was placed in a 5 ml polypropylene tube (BD Falcon, Franklin Lakes, NJ) and denatured with 100 µl of 0.1 M formic acid containing ~5 ng $^{13}C_2$-PFOA. Samples were vortexed for 1 min. Samples then received 1.0 ml of cold acetonitrile to precipitate proteins, were vortexed for 1 min and then centrifuged at 10,000 rpm for 2 min to pelletize proteins. An aliquot of the acetonitrile extract (200 µl) was combined with 200 µl of 2 mM ammonium acetate for LC/MS-MS analysis. All unknowns, replicates, method and matrix blanks, and quality assurance/quality control (QA/QC) samples were prepared in this fashion. The standard curve preparation was matrix-matched. Standards were prepared by spiking 25 µl of control Pel-Freeze CD1 mouse serum (Pel-Freeze Biologicals, Rogers, AR) into a 5 ml tube, then spiking in a corresponding mass of PFOA in methanol (0.250 - 1,250 ng) relating to 10 - 50,000 ng PFOA/ml serum. This was covered by two standard curve ranges (10 – 500 ng/ml) and (500 – 50,000 ng/ml). Standards were likewise treated as all other samples. Samples that did not fall in the lower standard curve range were re-run with a slightly modified method. The 25 µl serum sample was diluted with 2.0 ml of 0.1 M formic acid, and then 200 µl was sampled and added to 2.0 ml of acetonitrile containing ~62.5 ng of $^{13}C_2$-PFOA. This acetonitrile extract was then used to prepare the samples further as described above.

Samples were quantitated using a Waters Acquity UPLC coupled to a Quatro Premier XE (MS/MS) Waters Corp, Milford, MA). Samples were run in a batch as to include double blanks (solvent blank), a method blank, matrix blank (blank serum), standards, QC samples, replicates, and unknowns in sequence. Standards were run at the beginning and end of the analytical batch, and QC samples were interspersed in the analytical batch. QC samples consisted of pooled mouse serum spiked at six concentrations over the analytical range; the average accuracy of QC pooled samples was 94.1%, with a %RSD of < 10% for all samples (n = 18). PFOA was monitored via the transition 413-369 and 413-169 and for the $^{13}C_2$-PFOA 415-370. Samples were run using an isocratic (50:50) mobile phase consisting of 2 mM ammonium acetate aqueous solution with 5% methanol (Solvent A) and 2 mM ammonium acetate in acetonitrile (Solvent B). Samples were integrated using the equipment software and corrected, if necessary, by the operator.

Statistical analysis

All data are presented as mean ± standard deviation. Statistical analyses were performed with the SAS System (SAS Institute, Cary, NC). We performed a one-way repeated-measures analysis of variance (ANOVA) on dam and offspring BW by dose and a one-way ANOVA on organ weights, PFOA serum concentrations, and antibody titers by dose. When ANOVA indicated a statistically significant dose effect within operation, we made individual post hoc comparisons using Tukey's test and an LSMeans *t*-test. Statistical significance was determined using a P-value of 0.05.

Results

Dams

Weight gain of dams from the treated groups did not differ statistically from weight gain of dams given vehicle control water (Figure 1). However, weight gain between the 0.5 and 1 mg/kg groups did differ statistically; the 0.5 mg/kg group weighed approximately 10% more than the 1 mg/kg group at GD12 and GD19 ($P < 0.05$). The percentage of non-pregnant dams per group was about 30% (5/16) and only the 1 mg/kg group had a dam (1/16) that was pregnant but that did not deliver viable pups.

Pups

Litter sizes (3, 3.8, and 3.4 pups each, on average, for the 0, 0.5, and 1 mg/kg groups, respectively) and sex ratios were statistically equal across doses. From PND2-PND14, litter weights differed statistically by dose, but not by sex within dose (Figure 2). At PND2, the mean litter weight of both the 0.5 and the 1 mg/kg groups was 7-10% less relative to the control group ($p < 0.0001$). At PND7 and PND14, the 0.5 mg/kg group was equivalent in litter weight to the control group and the 1 mg/kg group still lagged behind the control group by 15% and 5%, respectively, by time.

At the time of organ collection for IgM (PND49; Table 1) and IgG (PND63), the absolute and relative mean weights of the adrenal gland, liver, spleen, or thyroid did not differ statistically by dose.

At the doses tested (0.5 and 1 mg/kg) and for the duration/timing of exposure (GD6-GD17), neither IgM (Figure 3) nor IgG (data not shown) titers were statistically affected by PFOA. Serum titers between control animals and dosed animals varied by less than 10%. In 20-day-old male pups, PFOA serum concentrations were 1,558 and 3,407 ng/ml (0.5 and 1 mg PFOA/kg groups, respectively; Figure 4A). At the time of serum collection for IgM titers, concentrations of PFOA in the serum were 200-300 fold higher in dosed animals relative to

control animals (Figure 4B). Two weeks later, at the time of serum collection for IgG titers, mean PFOA serum concentrations of treated animals were three to five times lower than the mean PFOA serum concentrations measured in serum collected for IgM evaluation (Figure 4B).

Discussion

Several studies have evaluated PFOA's immunotoxicity in adult rodent models and several have evaluated developmental toxicity, but to our knowledge, this is the first study to assess the potential DIT of PFOA in a rodent model. In this study, we evaluated T cell-dependent antibody responses (TDAR) in adult female offspring from C57BL/6 dams given 0.5 or 1 mg PFOA/kg of body weight from GD6-GD17. We chose to evaluate just TDAR as it is an immune response that is consistently impaired in adult mice after 10-15 days of exposure to 3.75-30 mg PFOA/kg (Yang et al., 2002; DeWitt et al., 2008). However, at the doses we administered, TDAR in C57BL/6 offspring appears to be unaffected by PFOA exposure when given to dams from GD6-GD17. Previous studies in adult rodent models orally exposed to PFOA indicate that TDAR is suppressed at doses from 3.75-30 mg PFOA/kg. These doses correspond to PFOA serum concentrations of 7.4×10^4 ng/ml (3.75 mg/kg) through 1.6×10^5 ng/ml (DeWitt et al., 2008) in adult female C57BL/6 mice dosed for 15 days. Serum concentrations in CD-1 male mice dosed for 14 days were analogous; 3 mg/kg given orally resulted in a serum PFOA concentration of 1.0×10^5 ng/ml (Loveless et al., 2006). In the current study, serum concentrations in female offspring evaluated for antigen-specific IgM antibody synthesis were 122 and 183 ng/ml for 0.5 and 1 mg PFOA/kg, respectively (Figure 3). It is our conclusion that doses of 0.5 and 1 mg PFOA/kg given to C57BL/6 dams for 11 days of gestation were not high enough or given long enough to raise offspring serum concentrations to a level sufficient to suppress TDAR.

To our knowledge only one other study has evaluated the DIT effects of PFAAs. Keil et al. (2008) reported that gestational exposure (GD1-GD17) to perfluorooctane sulfonate (PFOS) suppressed natural killer (NK) cell function and antigen-specific IgM antibody synthesis in adult male $B_6C_3F_1$ offspring. NK cell function was statistically suppressed by 1 and 5 mg PFOS/kg and IgM antibody production was statistically suppressed by 5 mg PFOS/kg. The Authors calculated a lowest observed adverse effect level (LOAEL) of 1 mg PFOS/kg for suppression of immune function after developmental exposure. Although Keil et al. (2008) did not report serum concentrations, PFOS may be a stronger immunosuppressant than PFOA or $B_6C_3F_1$ mice may be more sensitive to the DIT effects of PFAAs than C57BL/6 mice. In a related study with adult $B_6C_3F_1$ mice given PFOS via oral gavage for 28 days, serum concentrations of 91.5 ng/g (LOAEL = 0.05 mg PFOS/kg) were sufficient to suppress TDAR in male mice (Peden-Adams et al., 2008). The reported PFOA serum concentration associated with the LOAEL for suppression of TDAR in C57BL/6 adult female mice after 15 days of oral exposure was three orders of magnitude higher, i.e., 7.4×10^4 ng/ml (DeWitt et al., 2008). Strain and pharmacokinetic differences likely influence the ability of PFAAs to affect antibody production and to induce developmental toxicity.

When 5 mg PFOA/kg was given via oral gavage to CD-1 mice from GD1-GD17, neonatal survival decreased by about 20-30% relative to controls (Lau et al., 2006; Wolf et al., 2007). In an initial study (unpublished) in our laboratory, 5 mg PFOA/kg given to C57BL/6 mice via drinking water from GD6-GD17 decreased neonatal survival by 75% (6/8 litters died by PND6) and pups from only two litters survived until weaning. At weaning, body weights of male pups given 5 mg PFOA/kg were about 60% lower relative to body weights of control pups. At PND41, body weights of female pups given 5 mg PFOA/kg were ≈ 20% lower relative to body

weights of control pups. Therefore, this initial study indicated that because of overt developmental toxicity, 5 mg PFOA/kg was not an appropriate dose for evaluating DIT in C57BL/6 mice and that C57BL/6 mice appear to be more sensitive to the developmental effects of PFOA than CD-1 mice.

Our inability to detect DIT effects in C57BL/6 mice may also be related to differences in disposition or elimination in C57BL/6 mice relative to other strains of mice. In a study by Wolf et al. (2006) with CD-1 mice, serum concentrations in 6-wk-old female offspring exposed to 3 mg PFOA/kg from GD1-17 were 2,063 ng/ml (Wolf et al., 2007). In our study, serum concentrations in 7-wk-old female offspring exposed to 1 mg PFOA/kg from GD6-17 were 183 ng/ml. If a linear relationship for PFOA pharmacokinetics is assumed for low dose exposures (Rodriguez et al., 2009), our data indicate that C57BL/6 mice either accumulate or eliminate PFOA at a different rate than CD-1 mice. In a different study with 129S1/Sv1mJ mice, Abbott et al. (2007) reported serum PFOA concentrations of 9,860 ng/ml in weaning age male and female pups exposed to 1 mg PFOA/kg given via gavage from GD1-GD17. Serum PFOA concentrations in weaning age male pups from our study (not used for antibody responses) were 3,410 ng/ml; our data again indicate that strain differences may alter accumulation or elimination. Although our exposure duration was shorter than the studies by Wolf et al. (2007) and Abbott et al. (2007) by five days and we administered PFOA via drinking water rather than gavage, it is possible that C57BL/6 mice, at least for developmental exposures, have a different pharmacokinetic profile for PFOA than other strains for mice.

Conclusions

Doses of 0.5 and 1 mg PFOA/kg given to C57BL/6 dams for 11 days of gestation were not high enough or given long enough to raise offspring serum concentrations to a level

sufficient to suppress TDAR. However, other strains of mice are sensitive to the DIT of related perfluorinated compounds, adult C57BL/6 mice are sensitive to the immune effects of PFOA, and C57BL/6 offspring are sensitive to the developmental effects of PFOA when given during gestation, we plan an additional study to expose C57BL/6 dams a lower dose of PFOA for a longer duration. We anticipate that longer, lower dose exposures will more effectively mimic human exposures and give us a better idea of the potential DIT of PFOA.

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Declaration of Interests

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TABLE 1

Selected relative organ weights of female offspring (PND48) from dams exposed to PFOA from GD6 through GD17.

	Dose to Dam (mg PFOA/kg body weight)		
Organ	0	0.5	1
Adrenal	0.18 ± 0.03	0.20 ± 0.04	0.21 ± 0.04
Liver	48.01 ± 4.65	49.17 ± 6.59	50.84 ± 2.66
Spleen	4.88 ± 0.53	4.82 ± 0.36	5.26 ± 0.28
Thymus	4.50 ± 0.36	4.41 ± 0.41	4.88 ± 0.56

Data presented as mean ± standard deviation (N = 8 animals/dose). Means of organ weights do not differ statistically significantly by dose.

Figure Legends

Figure 1. Mean body weights (BW) of C57BL/6 dams exposed to PFOA from gestational day (GD) 6 through GD17 (mean ± standard deviation). Mean BW of the 0.5 and 1 mg PFOA/kg differed statistically at GD12 and GD19 (— $P < 0.05$). N = 16 dams/dose.

Figure 2. Mean litter weights of C57BL/6 pups from dams exposed to PFOA from gestational day (GD) 6 through GD17 (mean ± standard deviation). Litter weight of the 0.5 and 1 mg PFOA/kg groups was smaller relative to the control group at postnatal day (PND) 2; at PND7 and PND14, only the 1 mg PFOA/kg group was smaller relative to the control group (*$P < 0.05$). N = 10 litters/dose.

Figure 3. Mean SRBC-specific IgM antibody titers (mean ± standard deviation) in female offspring (PND48) from dams exposed to PFOA from GD6 through GD17. No statistical differences were detected among the dose groups. N = 8 animals/dose.

Figure 4. Mean serum PFOA concentrations (ng/ml; mean ± standard deviation) in **(A)** male offspring and **(B)** female offspring from dams exposed to PFOA from GD6 through GD17 (mean ± standard deviation; *$P < 0.05$). N = 8 animals/dose.