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Persistent alterations in immune cell populations and function from a single dose of perfluorononanoic acid (PFNA) in C57Bl/6 mice

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ABSTRACT

Perfluorononanoic acid (PFNA) is a perfluoroalkyl substance (PFAS) that is structurally related to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). Whereas PFOA and PFOS are known immunotoxicants, PFNA is less well characterized. Our previous study showed that PFNA has immunomodulatory effects on leukocyte populations and immune function. The present studies sought to determine whether, and to what degree, the immune system recovered 28 days after PFNA exposure. None of the parameters measured had fully recovered. A few parameters had partially recovered, including decreased spleen size and the decreased ratio of the $CD4^+/CD8^+$ double-positive population in thymus. The majority of effects of PFNA remained unchanged 28 days after exposure, including decreased proportion of intact thymocytes (as determined by FSC vs SSC), alterations in the ratios of immune cell populations in spleen and the $CD4^+$, $CD8^+$ and double-negative populations in thymus. Notably, PFNA markedly increased the TNFα response to LPS *in vivo*, and no recovery was evident 28 days after exposure. The effect of PFNA on $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ cells was more pronounced in females. The current study demonstrates that a single high dose exposure to PFNA (e.g. as might occur accidentally in an occupational setting) has long-lasting effects on the immune system.

1. Introduction

Perfluoroalkyl substances (PFAS's) are a family of chemicals that have unique water-repelling and oil-repelling characteristics. Because of these characteristics, these chemicals have numerous applications, including protective coatings for paper, cardboard, carpet, leather and textiles. In addition, PFAS's have been used in waterproofing products, floor polish, flame retardants, fire-fighting foams, and adhesives. PFAS's have also been used in the manufacturing of fluoropolymers, which have various uses including coatings for non-stick cookware and in the insulation of electrical wire (ATSDR, 2009; Prevedouros et al., 2006). In general, chemicals in this class have low water solubility, low volatility, are persistent in the environment, and bioaccumulate in animals. Widespread detection of numerous PFAS's has been observed in the serum of the U.S. population and in other countries (CDC, 2009; CDC, 2012). While serum levels of several PFAS chemicals appear to be declining in the U.S. and elsewhere, perfluorononanoic acid (PFNA) is a notable exception. Previous studies indicate that serum levels of PFNA doubled over a six year period within the U.S., but the reason for this is not currently clear (Calafat et al., 2007).

Toxicity concerns with respect to the PFAS's began to emerge when it was found that the ammonium salt of perfluorooctanoic acid (PFOA) has numerous toxic effects in animal models, including hepatotoxicity, alterations in the immune system and in development. With respect to humans, PFOA and perfluorooctane sulfonate (PFOS) exposure have been associated with decreased birth weight (Apelberg et al., 2007). Similarly, PFNA has also been shown to have adverse biological effects. In animal models, PFNA has been shown to disrupt glucose metabolism (Fang et al., 2012), impair neonatal survival and development (Wolf et al., 2010), induce hepatomegaly

Abbreviations used: PFAS, Perfluoroalkyl substances; PFNA, Perfluorononanoic acid; PFOA, Perfluorooctanoic acid; PFOS, Perfluorooctane sulfonate.

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and hepatic peroxisomal β-oxidation (Kudo et al., 2006) and cause immunotoxicity (Fang et al., 2008; Rockwell et al., 2013).

With respect to immunotoxicity, daily PFNA administration over the course of 14 days caused splenic and thymic atrophy and inhibition of cytokine production in BALB/c mice, an animal model that tends to be Th2-skewed (Fang et al., 2008). In C57BL/6 mice, an animal model that tends to be more Th1-skewed, we found PFNA caused similar effects. A single dose of PFNA (0.1 mmol/kg) caused marked splenic and thymic atrophy and an altered balance of immune cell populations in the spleen and thymus 14 days after administration (Rockwell et al., 2013). The dose of PFNA (0.1 mmol/kg) was chosen to correlate with a relatively high dose as might occur in an accidental and/or occupational exposure. The purpose of the present study was to determine whether there was any recovery from these effects at a later time-point, specifically, 28 days after PFNA administration. This time-point was chosen based on the time to recovery of toxic and immunomodulatory effects of other PFAS's as determined by previous studies from our lab (unpublished observations) and other investigators.

2. Materials and methods

2.1. Materials

PFNA (free acid form; 97% purity; M.W. 464 g/mol) was purchased from Sigma-Aldrich Co. (St Louis, MO).

2.2. PFNA administration to mice

Eight-week-old adult male and female C57BL/6 J mice were purchased from Jackson Laboratories (Bar Harbor, Maine), and housed according to AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) guidelines. Adult C57BL/6 male ($n = 5$) and female ($n = 5$) mice were administered a single dose of PFNA (0.1 mmol/kg of body weight) by intraperitoneal (i.p.) injection. Control mice were treated i.p. with the vehicle, propylene glycol:water (1:1, v/v). After four weeks, the spleen, thymus, and liver were collected from each mouse. For studies with LPS, it was administered 4 weeks after PFNA exposure. The mice were given a single i.p. dose of LPS (1 mg/kg) or vehicle (sterile saline). One hour and 30 min after LPS administration, blood was collected from each animal and TNFα concentrations were quantified. The animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kansas Medical Center and/or Michigan State University.

2.3. Flow cytometry

Splenocytes and thymocytes were isolated by manually dissociating spleens and thymus respectively using glass slides. Freshly-isolated splenocytes or thymocytes were washed and resuspended in FACS buffer (PBS, 1% FCS). The cells were then incubated with anti-CD4/PerCP (RM4-5, 0.4 μg/ml), anti-CD8/APC (Ly-2, 0.4 μg/ml), anti-CD19/PE (MB19-1, 0.4 μg/ml), and/or anti-CD14/FITC (Sa2-8, 1 μg/ml) for 30 min at 4 °C, after which the cells were washed and fixed with BD Cytofix fixation buffer (BD Biosciences, San Jose, CA). The fluorescence was then detected and quantified with a BD™ FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Spectral overlap was manually compensated by a trained flow cytometrist. The data were analyzed using FlowJo 7.5 software (Tree Star, Inc., Ashland, OR).

2.4. TNFα ELISA

TNFα levels in the serum were quantified by a commercially available mouse TNFα ELISA kit according to the manufacturer's protocol (Ebioscience, San Diego CA). The relative absorbance of each sample was quantified on a Bio-Tek μQuant microplate reader (Highland Park, VT). The TNFα concentrations were calculated using a 4-parameter standard curve.

2.5. Statistical analysis

The mean ± standard error was determined for each treatment in the individual experiments. Homogeneous data were evaluated by two-way parametric analysis of variance. When significant differences were observed, the Holm-Sidak post-hoc test was used to compare treatment groups to the vehicle (VH) control using SigmaStat 3.01a software from Systat Software, Inc. (Chicago, IL).

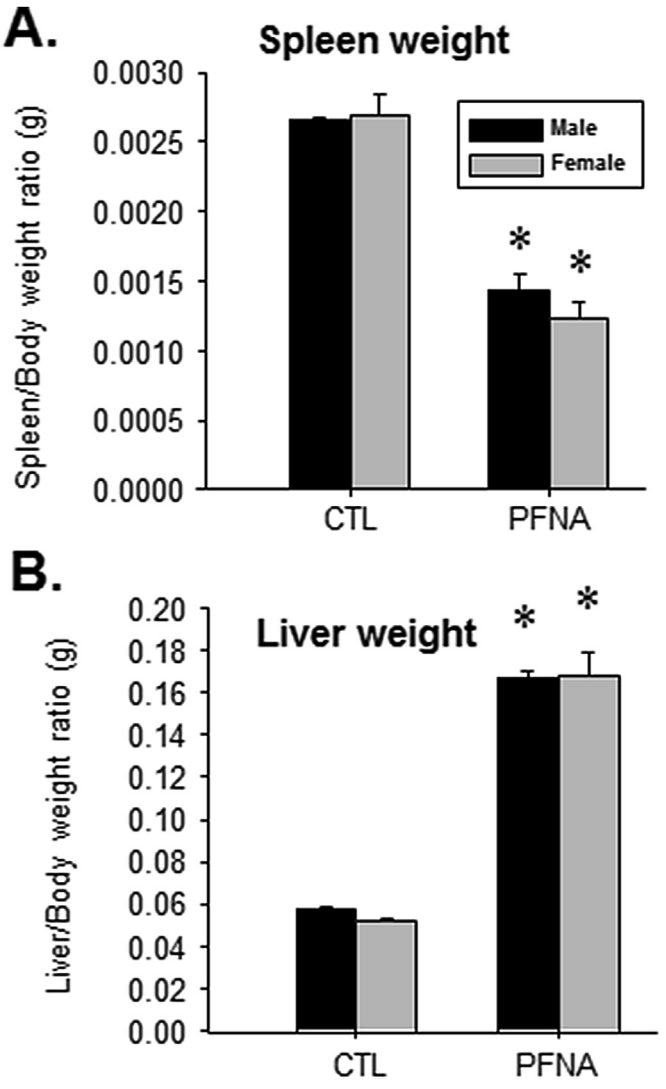

Fig. 1. The relative organ weights of mice four weeks after PFNA administration. The ratios of (A) spleen and (B) liver to body weight were measured four weeks after PFNA administration. The data are presented as the mean ± SE. * denotes $p < 0.05$ as compared to the vehicle control (CTL) for the same gender.