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Exposure to the Immunosuppresant, Perfluorooctanoic Acid, Enhances the Murine IgE and Airway Hyperreactivity Response to Ovalbumin

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These studies were conducted to investigate the role of dermal exposure to perfluorooctanoic acid (PFOA), a known immunosuppressant, on the hypersensitivity response to ovalbumin (OVA) in a murine model of asthma. PFOA has had widespread use as a carpet and fabric protectant. BALB/c mice were exposed dermally, on the dorsal surface of each ear, to concentrations of PFOA ranging from 0.01 to 1.5% (applied dose 0.25–50 mg/kg) for 4 days. In hypersensitivity studies, mice were also ip injected with 7.5 μg OVA and 2 mg alum on days 1 and 10 and in some studies challenged with 250 μg OVA by pharyngeal aspiration on days 17 and 26. Following exposure to PFOA, an increase in liver weights and a decrease in thymus and spleen weights and cellularities were observed. Similar immunomodulatory trends were demonstrated in mice coadministered PFOA and OVA. Compared to the OVA alone–exposed animals, an increase in total IgE was demonstrated when mice were coexposed to OVA and concentrations of PFOA ranging from 0.75 to 1.5%, while the OVA-specific IgE response peaked with 0.75% PFOA coexposure ($p \leq 0.05$). OVA-specific airway hyperreactivity was increased in the 1.0% PFOA coexposed group ($p \leq 0.05$), with an increased pleiotropic cell response characterized by eosinophilia and mucin production, in animals coexposed to concentrations of PFOA up to 1.0%, as compared to the OVA alone–exposed animals. In a murine model, PFOA was demonstrated to be immunotoxic following dermal exposure, with an enhancement of the hypersensitivity response to OVA, suggesting that PFOA exposure may augment the IgE response to environmental allergens.

Key Words: **perfluorooctanoic acid; ovalbumin; airway hyperreactivity; IgE.**

Perfluorooctanoic acid (PFOA) is an organic fluorochemical once used primarily as a surfactant in the polymerization of chemicals including fluoroacrylic esters, fluoropolymers, and fluoroelastomers. Commercial applications pertinent to these processes include manufacturing of flame retardants and extinguishers, surfactants, waxes and gloss finish enhancers, and water repellants in fabrics, (Kudo and Kawashima, 2003; Starkov and Wallace, 2002) with the main usage being carpet and fabric protectants. Additional sources of PFOA are fluorotelomer alcohols, which can be biochemically modified to become PFOA (Ellis *et al.*, 2003; Martin *et al.*, 2002). Fluorotelomer alcohols are incorporated into polymers for strain repellency properties for clothing, carpets, and textile products (Martin *et al.*, 2004). Fluorotelomer products are also used in a variety of manufacturing processes, such as waxes, graphic arts, metals, caulks, coatings, polymers, polishes, adhesives, electronics, and paints (Ellis *et al.*, 2003).

The hydrolytic half-life of PFOA in the environment is estimated to be greater than 97 years and the biological half-life in humans is reported to be 4.37 years (Kudo and Kawashima, 2003). As a result, perfluorinated acids have been found in the environment and in various species of animals including mink, bald eagles, tuna, dolphins, aquatic organisms, and whales (Giesy and Kannan, 2001; Kannan *et al.*, 2001, 2002a,b,c; Nakata *et al.*, 2006) Organic fluorines have been identified not only in the blood of occupationally exposed individuals (Kudo and Kawashima, 2003; Ubel *et al.*, 1980) but in the general population as well (Emmett *et al.*, 2006a,b; Olsen *et al.*, 1998, 2003a). Occupational surveillance has demonstrated an increase in PFOA levels in the serum of individuals with and without workplace exposure, with ambiguous reports of human toxicity (Gilliland and Mandel, 1996; Kudo and Kawashima, 2003; Olsen *et al.*, 1998, 2003b).

Numerous animal studies have demonstrated PFOA-induced organ (liver, thymus, spleen) and systemic (body weight loss) toxicity after dermal and oral exposure. PFOA has also been shown to be immunotoxic in murine models. Mice fed a diet containing 0.02% PFOA for 10 days had a 50 and 90% decrease in the total number of splenocytes and thymocytes, respectively (Yang *et al.*, 2000). In a subsequent study, animals fed a diet containing PFOA had a decrease in both splenic IgM and IgG plaque forming cells and decreased serum levels of IgM and IgG in response to immunization with horse red blood

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cells (Yang *et al.*, 2002). Similar results have been generated in our lab after dermal exposure to PFOA (unpublished data).

The incidence of asthma and allergic diseases has increased in the past several decades with a greater percent increase observed in children than any other group (Mannino *et al.*, 2002). As one of the major uses of PFOA has been in carpet and fabric protectants, the potential exists for exposure of children through dermal as well as hand to mouth contact and adults through both environmental and occupational exposures (Begley *et al.*, 2005) (Kubwabo *et al.*, 2005). Although the mechanisms of asthma are not fully understood, it has been proposed that the balance between regulated and deregulated immune responses may be important (Wills-Karp *et al.*, 2001). Therefore, due to PFOA's potential for both environmental and occupational exposure, its long half-life, and known immunotoxic effects, these studies were undertaken to determine if exposure to PFOA would modulate the allergic response to ovalbumin (OVA) in a well studied and characterized murine model of asthma.

MATERIALS AND METHODS

Animals

Female BALB/c mice, obtained from Taconic Laboratories (Germantown, NY) at 6–8 weeks of age, were allowed to acclimate for at least 1 week before the start of each study. The animals were housed five per cage in ventilated plastic shoe box cages with hardwood chip bedding, fed modified NIH-31 6% irradiated rodent diet (Harlan Teklad item #7913), and given tap water from water bottles *ad libitum*. A standard light/dark cycle was maintained on 12-h intervals. The room temperature was maintained between 65 and 78°F and the humidity between 20 and 60%. Cages were cleaned and sanitized weekly. Mice were individually identified and assigned to homogenous weight groups prior to the first exposure. Animals were weighed immediately before sacrifice in all studies. The NIOSH animal research program facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Test Articles

Chicken egg albumin (OVA; Grade V; purity > 98%), aluminum potassium sulfate dodecahydrate (ALUM; ACS Reagent), acetone (purity 100%), phosphate-buffered saline (PBS), pH 7.4, and PFOA (purity > 96%) were all purchased from Sigma-Aldrich (St Louis, MO). PFOA was prepared in acetone at concentrations ranging from 0.01 to 1.5% (wt/vol; 0.25–50 mg/kg/dose). OVA was either prepared at 7.5 μg in 100 μl sterile PBS with 2 mg ALUM for ip injection or at 250 μg in 50 μl sterile PBS for pulmonary exposure via pharyngeal aspiration.

Animal Exposures

Toxicity study. Mice were exposed on the dorsal surface of each ear with 25 μl per ear of acetone control vehicle or a concentration of PFOA ranging from 0.01 to 1.5% on days 1–4. Animals were manually restrained until acetone was evaporated and then returned to their cage. Mice were sacrificed via CO_2 inhalation 6 days after final exposure.

Hypersensitivity studies. Mice were exposed on the dorsal surface of both ears with 25 μl per ear of acetone vehicle control or a concentration of PFOA ranging from 0.25 to 1.5% on days 1–4 and injected ip with either 2.0 mg ALUM or 7.5 μg OVA and 2.0 mg ALUM in 100 μl sterile PBS on days 1 and 10.

TABLE 1
Organ Weights in BALB/c Mice Following 4-Day Dermal Exposure to PFOA

Exposure group	Liver weight (g)	Thymus weight (mg)	Spleen weight (mg)
Acetone	0.99 ± 0.02	48.80 ± 3.89	81.80 ± 4.25
0.01% PFOA (0.25 mg/kg)	1.02 ± 0.04	48.00 ± 3.74	80.00 ± 4.41
0.1% PFOA (2.5 mg/kg)	1.26 ± 0.08	53.20 ± 4.16	81.20 ± 4.87
0.25% PFOA (6.25 mg/kg)	1.51 ± 0.05**	57.80 ± 2.90	76.60 ± 6.14
0.5% PFOA (12.5 mg/kg)	2.13 ± 0.07**	54.50 ± 3.86	73.50 ± 3.86
1.0% PFOA (25 mg/kg)	2.20 ± 0.07**	43.75 ± 4.55	59.25 ± 5.15*
1.5% PFOA (50 mg/kg)	2.34 ± 0.15**	28.75 ± 5.45*	55.00 ± 4.79**

Note. Animals were sacrificed 6 days after final exposure. Data presented are the group mean organ weights ± SE; $n = 5$ for all exposure groups.

*$p \leq 0.05$ and **$p \leq 0.01$ indicated statistical significant as compared to the acetone control analyzed by Dunnett's post test.

Four days following the final OVA exposure, mice were sacrificed, and blood was obtained via cardiac puncture (IgE evaluation studies). For airway hyperreactivity evaluation, mice were dosed according to the above protocol and challenged with 250 μg OVA in 50 μl by pharyngeal aspiration on days 19 and 26. Mice were sacrificed 24 h after final pharyngeal aspiration.

PFOA Modulation of the NonSpecific and Antigen-Specific Airway Hyperreactivity Response in OVA-Sensitized Mice

Airway hyperreactivity was assessed in mice as described previously (Howell *et al.*, 2002) with minor modifications. Briefly, for nonspecific challenge on day 12 after initial exposure, mice were placed in Buxco whole-body plethysmography chambers and baseline enhanced pause (PenH) values were obtained over a 5-min period. Mice were then challenged by nebulizing increasing concentrations (10, 25, and 50 mg/ml) of methacholine (MCH; Sigma-Aldrich) into the chambers. At each concentration of MCH, PenH was assessed over a 5-min period with MCH exposure occurring for the first 3 min followed by 2 min of fresh air. Mice were tail bled on day 14 for total IgE analysis. On days 19 and 26, following light anesthesia with isoflurane (Abbott Laboratories, 99.9%), mice were challenged with either 50 μl sterile PBS or 250 μg OVA/50 μl sterile PBS via pharyngeal aspiration using the method described by Rao *et al.* (2003). Prior to challenge on day 26, a 5-min baseline PenH was assessed for each animal via Buxco whole-body plethysmography. Following challenge, animals were immediately returned to the chambers and PenH was calculated every 30 s over the next 5 h. Experimental values minus mean baseline PenH value for each animal were used in the calculation of area under the curve (AUC) for the entire evaluation period using Graph-Pad Prism (San Diego, CA). An animal was considered a positive responder if the PenH value was equal to or greater than 1.6. Twenty-four hours after the final pharyngeal aspiration, mice were sacrificed, and blood was collected by transection of the abdominal aorta. Lungs were fixed by perfusion of 1 ml 10% buffered formalin via the trachea and placed in 10 ml of 10% buffered formalin for histopathology analysis. Eosinophil staining was performed using a modified Hansel's protocol, where sections were stained for 1 min with Mayers hematoxylin in place of 0.5% methylene blue (Matsuse *et al.*, 1991). Hematoxylin and eosin (H&E) and Alcian Blue/PAS (mucin) staining were also performed. Histopathology evaluations were performed on sections of the left lung from each animal.

Total Serum IgE

Total IgE was quantified by ELISA as described by Manetz and Meade (1999) with minor modifications. Briefly, 96-well flat bottom plates (Dynatec Immulon-2, Chantilly, VA) were coated with 0.2 μg B1E3 rat anti-mouse IgE

TABLE 2
BALB/c Toxicity Evaluation Following PFOA and OVA/PFOA Exposure over a 14-Day Period

PFOA	OVA	*n*	Body weight (g)	Liver weight (g)	Spleen cellularity × 10^6	Thymus cellularity × 10^6	Spleen weights (mg)	Thymus weights (mg)
—	+	15	19.72 ± 0.27	1.06 ± 0.02	159.10 ± 18.92	96.63 ± 6.31	113.6 ± 3.42	63.97 ± 3.09
0.5%	+	15	20.01 ± 0.43	2.06 ± 0.06**	99.99 ± 11.01**	104.20 ± 6.97	95.43 ± 3.08**	59.77 ± 2.63
0.75%	+	5	17.78 ± 1.21	1.82 ± 0.14**	40.16 ± 2.39**	45.86 ± 12.56	96.10 ± 8.32	41.96 ± 11.62*
1.0%	+	14	18.21 ± 0.54	2.18 ± 0.06**	89.89 ± 12.25**	44.97 ± 6.69**	76.89 ± 6.42**	35.83 ± 3.67**
1.5%	+	10	17.29 ± 0.39*	2.33 ± 0.07**	88.21 ± 12.96*	30.52 ± 11.64**	51.15 ± 2.57**	22.96 ± 4.37**

Note. Data presented are group means ± SE, with *n* indicating the number of animals represented in an exposure group. Cellularities presented are the absolute number of cells × 10^6 (calculated based on cell counts multiplied by percent of total cells).

*Indicates $p \leq 0.05$, while **indicates $p \leq 0.01$ as analyzed by Dunnett's post test with comparisons made against the OVA-alone groups (7.5 μg).

and incubated overnight at 4°C. Serum samples, PharMingen standard (5000 ng/ml, mouse IgE anti-TNP; San Diego, CA), and internal control (2μg/ml at a 1:40 dilution, mouse IgE anti-DNP) were serially diluted (1:2) through eight wells and added to the coated plates. Biotin-conjugated rat anti-mouse IgE (0.2 μg, clone R35-92; PharMingen) was used as the secondary antibody. Strepavidin-alkaline phosphatase and *p*-nitrophenyl phosphate were sequentially added to the plates (both obtained from Sigma Chemicals). Absorbance was determined using a Spectramax Vmax plate reader (Molecular Devices, Sunnyvale, CA) at 405–650 nm. Data analysis was performed using IBM Softmax Pro 3.1.1 (Molecular Devices), and the IgE concentrations for each sample were interpolated from a standard curve using multipoint analysis. The hybridoma cell lines for B1E3 and mouse IgE anti-DNP antibodies were kindly provided by Dr Daniel Conrad (MCV/VCU, Richmond, VA).

OVA-Specific IgE ELISA

The OVA-specific IgE ELISA was conducted according to Hogan *et al.* (2003). Plates were coated with 200 ng monoclonal rat anti-mouse IgE (PharMingen) and then blocked with PBS/1% skim milk. Samples (1:10 and 1:40) were then added and serially diluted 1:2 through eight wells. OVA (2.5 mg), rabbit anti-OVA-HRP conjugate (10 mg/ml, 1:8000 dilution, Rockland Immunochemicals, Gilbertsville, PA), and tetramethylbenzidine (Sigma, St Louis, MI) were sequentially added. Absorbance was determined using a Spectramax Vmax plate reader (Molecular Devices) at 650 nm. Mouse monoclonal anti-OVA IgE (500 ng/ml, Serotec, Oxford, UK) was used to generate a standard curve. Data analysis was performed using the IBM Softmax

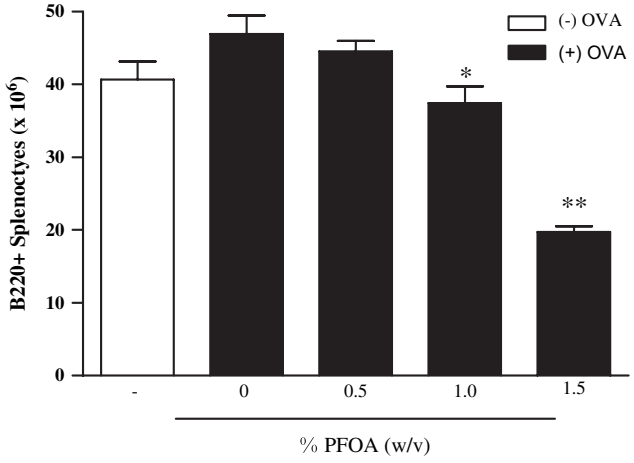

FIG. 1. Evaluation of $B220^+$ splenocytes in BALB/c mice after exposure to OVA and increasing concentrations of PFOA following sacrifice on day 27. Data represent the group mean ($n = 5$) absolute number of cells × 10^6 ± SE based on cell counts × percentage of total cells. Bars represent group mean ± SE. *Represents $p \leq 0.05$ and ** represents $p \leq 0.01$ as compared to the OVA- (7.5 μg) only exposure group.

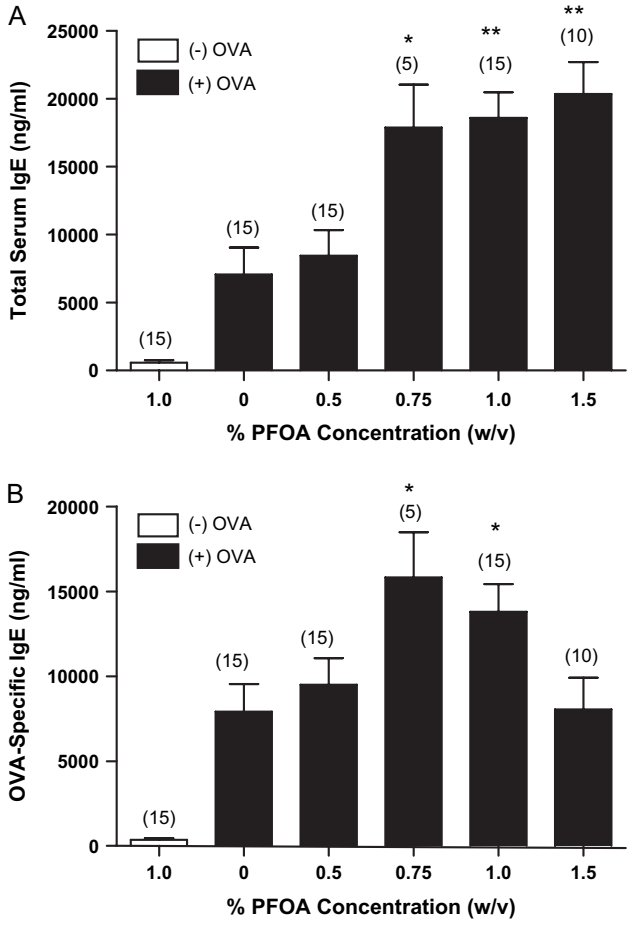

FIG. 2. Evaluation of the IgE response in BALB/c mice after coexposure to OVA and increasing concentrations of PFOA over a 14-day period. Graphs represent (A) total serum IgE and (B) OVA-specific serum IgE responses. Bars represent group mean ± SE. Numbers in parentheses overlying bars represent the number of animals per exposure group. PFOA and OVA-exposed groups were compared to OVA-alone (7.5 μg) exposure group. *Represents $p \leq 0.05$ as compared to the OVA control. Results are based on the combined data of three studies.

TABLE 3
Total and Antigen-Specific Serum IgE Response Following PFOA and OVA Exposure

		IgE Serum ELISA (ng/ml)			
PFOA	OVA	Day 14 Total IgE[a]	Day 14 OVA-specific IgE[b]	Day 27 total IgE[a]	Day 27 OVA-specific IgE[a]
−	−	509 ± 87.96	200 ± 112	406 ± 24	248 ± 159.0
+	−	1219 ± 151	150 ± 150	816 ± 253	43 ± 43.00
−	+	6031 ± 1785	1442 ± 833	7514 ± 2180	3487 ± 718.2
0.5%	+	7542 ± 2053	2239 ± 1534	13,596 ± 3752	5540 ± 1724
0.75%	+	6364 ± 1542	1185 ± 410	11,184 ± 1082	4747 ± 658.0
1.0%	+	35,933 ± 20,516*	6412 ± 2952*	19,994 ± 2571*	5697 ± 1721
1.5%	+	20,195 ± 4483	3195 ± 967	15,525 ± 4452[b]	4730 ± 1998[b]

Note. Data represented are total IgE and OVA-specific IgE ± SE.
[a]Designates $n = 5$ for the appropriate end point.
[b]Designates an n of 4 for the exposure group.
Statistical significant of groups is compared to OVA control dose group (IP [7.5 μg] and pulmonary [250 μg] administered), where $*p \leq 0.05$.

Pro 3.1.1, and the OVA-specific IgE concentrations for each sample were interpolated from the standard curve using multipoint analysis.

Phenotypic Analysis

After sacrifice, spleens and thymi were excised from the exposed mice and placed in 3-ml PBS, and single-cell suspensions were prepared. Approximately 1×10^6 cells per sample were added to the wells of 96-well u-bottom plates. Cells were stained using monoclonal antibodies against B220 (PE; clone RA3-6132), CD3e (FITC; 145-2C11), CD4 (FITC; clone RM4-5), CD8a (PE; clone 53-6.7), and isotype controls. The Fc block used was anti-mouse CD32/16 (clone 2.4 G2). All antibodies were purchased from PharMingen. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA), gating on viable non–red blood cells with results expressed as absolute number of cells (Manetz and Meade, 1999).

Statistics

The structure of these experiments was a completely randomized design, and the treatment structure utilized a one-way layout with animals randomly assigned to a vehicle control, test article, or positive control group. Differences were considered significant if $p \leq 0.05$ as compared to the vehicle control. Comparisons of end points between the control group and each treatment level were carried out using a one-way ANOVA to determine the overall effect with Dunnett's test (Dunnett and Crisafio, 1955) to determine differences between treatment group and control. If the assumptions were not able to be met by parametric analysis, the nonparametric Kruskal-Wallis k-sample test was utilized followed by the Mann-Whitney *U*-test for pairwise comparisons with the control. For combined data, analyses were generated using SAS/STAT software, Version 9.1 of the SAS System for Windows. A generalized randomized complete block design was utilized in PROC MIXED by incorporating the multiple replicated experiments as a random variable. Dunnett's tests were used to compare treatment groups to the control group.

When comparisons were made between two exposure groups only, an unpaired, two-tailed *t*-test was used, with values considered statistically significant at $p \leq 0.05$. Linear trend analysis was performed to determine if PFOA exposure had a dose-responsive effect on specified end points. Differences were considered significant if $p \leq 0.05$ as compared to the acetone vehicle or OVA control.

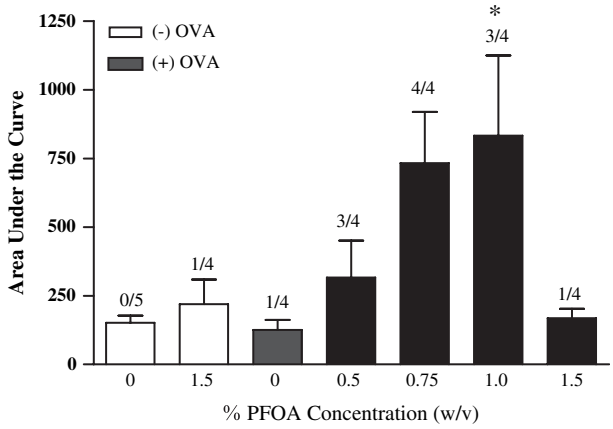

FIG. 3. Evaluation of OVA-specific airway hyperreactivity in BALB/c mice following coexposure to OVA and increasing concentrations of PFOA on day 26. Data represents the group mean AUC ± SE for all animals over the 5-h time course. The numbers above each bar represent the number of animals per group exhibiting a late-phase airway hyperreactivity response following challenge with OVA. PFOA and OVA-exposed groups were compared to OVA control dose group (IP [7.5 μg] and pulmonary [250 μg] administered), where $*p \leq 0.05$, and $**p \leq 0.01$.

RESULTS

Dermal Range Finding Studies

Initial studies were conducted to evaluate the toxicity of PFOA following dermal exposure in BALB/c mice and to select doses to be used in subsequent immunotoxicology studies. Animals were exposed on the dorsal surface of the ears for four consecutive days with concentrations of PFOA ranging from 0.01 to 1.5%. Mice were sacrificed on day 10, 6 days after final exposure and select organ weights were recorded (Table 1). There were no significant differences in body weights between the acetone control and exposure groups (data not shown). No signs of inflammation were noted at the site of application. Animals exhibited an ($p \leq 0.01$) increase in liver weight, reaching statistical significance in the 0.25% exposure group (1.51 ± 0.05 g) as compared to acetone control (0.99 ± 0.02 g) with a mean liver weight of 2.34 ± 0.15 g in animals of the 1.5% dose group. Conversely, a decrease was demonstrated in both the thymus ($p \leq 0.05$) and spleen ($p \leq 0.01$) weights of animals exposed to PFOA. A 41% decrease in thymus weight and a 33% decrease in spleen weight were observed in the 1.5% exposure group. Thymus and spleen cellularites were also decreased after PFOA exposure when compared to acetone control (data not shown).

Toxicity in Animals Coexposed to OVA and PFOA

Animals were coexposed to OVA and increasing concentrations of PFOA. Only animals coexposed to the highest concentration of PFOA (1.5%) and OVA had a significant decrease ($p \leq 0.05$) in body weight (17.29 ± 0.39 g) as compared to the OVA control animals (19.72 ± 0.27 g; Table 2).

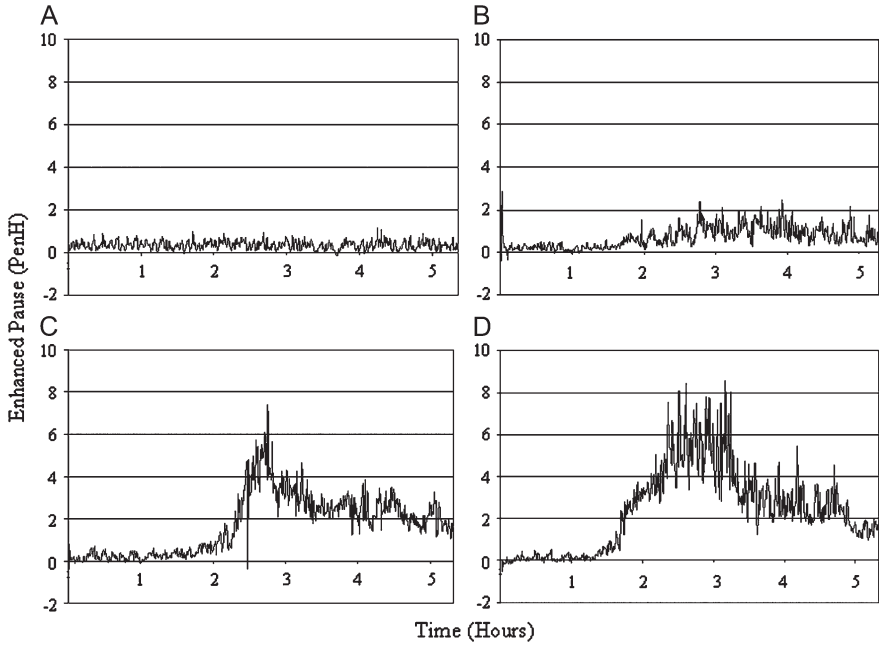

FIG. 4. Representative airway responses from OVA and OVA/PFOA-coexposed animals. Lines represent PenH values over a 5-h time period from animals exposed to (A) acetone control (PFOA, OVA), (B) OVA alone, (C) 0.75% PFOA and OVA, and (D) 1.0% PFOA and OVA.

Animals coadministered OVA, and all concentrations of PFOA exhibited increased ($p \leq 0.01$) liver weights as compared to the OVA only–exposed groups (Table 2). As seen in mice exposed to PFOA alone (Table 1), mice coexposed to PFOA and OVA demonstrated a decrease in mean spleen and thymus weights and cellularity (Table 2). These results are based on the combined data of three studies.

Phenotypic Analysis

A decrease in total spleen and thymus cellularity was demonstrated in animals coexposed to OVA and PFOA as compared to the OVA-control group (Table 2). The lowest observable effect level was 0.5% for the spleen and 1.0% for the thymus. Differences in the absolute numbers of $CD3^+$ T cells or T-cell subsets ($CD4^+$, $CD8^+$, or $CD4^-8^-$) in the thymi or spleens of coexposed animals did not reach statistical significance (data not shown). A PFOA dose-responsive decrease ($p \leq 0.00001$) in $B220^+$ splenocytes was observed in PFOA and OVA-coexposed animals as compared to the OVA control group (Fig. 1). No differences in B220+ numbers were observed between the acetone control (OVA) and OVA control (data not shown)

Despite Thymic and Splenic Toxicity, PFOA Enhanced the IgE Response Induced by OVA

Although studies have demonstrated a decrease in the IgM and IgG response to antigen following exposure to PFOA, the IgE response to allergens has not previously been evaluated. For the 14-day study, serum IgE levels in animals exposed to PFOA (1%) alone (1107 ± 322 ng/ml) were within the historical control data range for naive and acetone-exposed animals in our laboratory. Increases in total (17,875 ± 3178 ng/ml) and OVA-specific (15,831 ± 2640 ng/ml) IgE were demonstrated in the 0.75% PFOA and OVA-exposed group as compared to the OVA alone–exposed group (7081 ± 1970 ng/ml and 7905 ± 1637 ng/ml, respectively; Figs. 2A and 2B). The total serum IgE plateaued between concentrations of 0.75 and 1.5% PFOA with coexposure to OVA (7.5 μg), while the OVA-specific IgE peaked at 0.75% PFOA and OVA and then decreased when groups were exposed to concentrations of PFOA ranging from 0.75 to 1.5% (Figs. 2A and 2B). No differences were observed between the acetone control (PFOA, OVA) and PFOA- (1%) alone groups. These results are based on the combined data of three studies.

The Antigen-Specific Airway Hyperreactivity Response to OVA Is Enhanced Following CoExposure to PFOA

As the total and OVA-specific IgE responses were increased following coexposure to OVA and PFOA after a 14-day period, further studies were conducted to determine if PFOA exposure modulated nonspecific or antigen-specific airway hyperreactivity responses (AHRs) in OVA-sensitized animals. No significant differences in nonspecific AHR between the OVA control group and animals coexposed to OVA and increasing concentrations of PFOA were observed following MCH challenge on day 12 after initial exposure (data not shown).

TABLE 4
Inflammatory Responses in the Lungs of OVA and PFOA-Exposed Mice

Exposure regiment		Pleiotropic inflammatory response		Eosinophil infiltration		Mucin	
PFOA	OVA	No. of mice	Score	No. of mice	Score	No. of Mice	Score
−	−	5/5	−	5/5	−	5/5	−
+	−	5/5	−	5/5	−	5/5	−
−	+	3/5	+	5/5	+	2/5	#
		2/5	++			3/5	##
0.5%	+	2/5	+	2/5	+	1/5	–
		1/5	++	2/5	++	2/5	##
		2/5	+++	1/5	+++	2/5	###
0.75%	+	1/5	+	1/5	+	1/5	#
		2/5	++	4/5	++	1/5	##
		2/5	+++			3/5	###
1.0%	+	1/5	+	1/5	+	1/5	#
		4/5	++	4/5	++	4/5	###
1.5%	+	1/4	+	1/4	+	1/4	–
		2/4	++	2/4	+/++	2/4	####
		1/4	+++	1/4	++	1/4	

Numbers of mice demonstrating the indicated response (−, +, ++, or +++) for either pleiotropic inflammatory cells (including macrophages, neutrophils, giant cells, and lymphocytes) or eosinophils in the interstitial, perivascular, and peribronchular areas of the left lung, where −, +, ++, or +++ indicates no, mild, moderate, and high levels of cells present, respectively. Scores for mucin represent the following: no increase in mucin in cells or mucous in airways (−), increased mucin in cells but not in airways (#), increased mucin in cells and mucous present in airways (##), and increased mucin present in cells expressing a greater cell height into the airway and width, with mucous present in airways (###). Mucin evaluation was scored in the medium and large airways of the left lung. PFOA- and OVA-exposed groups were compared to OVA alone–exposed mice (IP [7.5 μg] and pulmonary [250 μg] administered).

Total IgE analysis on day 14 demonstrated an increase ($p \leq 0.05$) in total serum IgE in the PFOA (1.0%) and OVA- (7.5 μg) coexposed group (35,933 ± 20,516 ng/ml) as compared to the OVA control group (6031 ± 1785 ng/ml). Although not statistically significant due to individual variability, animals in the 1.5% coexposure group had a mean total IgE of 20,195 ± 4483 ng/ml (Table 3). No differences in total or specific IgE levels were observed between the PFOA (1.5%) control and acetone control (PFOA, OVA).

Animals were then dosed with either PBS or OVA by pharyngeal aspiration on day 19 to sensitize the lungs and then challenged with OVA by pharyngeal aspiration on day 26 and monitored over the following 5.3 h for changes in antigen-specific AHR. PenH values for the entire period were plotted for each group, and the AUCs were calculated. A trend toward increasing antigen-specific AHR was observed in animals which had been coexposed to OVA and increasing concentrations of PFOA up to 1.0%. An increase in the number of animals per group responding also increased with increasing concentrations of PFOA up to 1.0%. A significant ($p \leq 0.05$) increase in AUC was demonstrated in the 1.0% PFOA/OVA-coexposed group (832.4 ± 292.4) as compared to the OVA alone–exposed group (296.7 ± 98.5; Fig. 3). No differences were observed between the acetone control and PFOA- (1.5%) exposed mice. Representative AHR profiles of mice from the acetone control, OVA alone, 0.75% PFOA/OVA, and 1.0% PFOA/OVA-exposed groups are shown in Figure 4, with 0/5, 1/4, 4/4, and 3/4 animals responding from each exposure group, respectively (Fig. 3). On day 27, increases in total IgE levels were observed in all groups coexposed to PFOA and OVA as compared to the OVA-alone exposure group. By day 27, the OVA-specific IgE response plateaued and was not statistically elevated over the OVA control group (Table 3). At the conclusion of the AHR study, no significant differences were seen in body weights of PFOA-exposed animals as compared to controls (data not shown).

Histopathology

Histopathologic evaluation of lungs on day 27 of animals used in the AHR study identified a pleiotropic inflammatory cell response with primarily macrophage and eosinophil cell infiltration in all mice exposed to OVA by pharyngeal aspiration. The severity of the response appeared to increase with increasing concentrations of PFOA (Table 4). Representative sections of the left lung stained with H&E and Hansel's are shown in Figure 5. The response was characterized by massive infiltration of eosinophils and to a lesser extent macrophages into the interstitial, peribronchiole, and perivascular areas. Other cell types involved in the response included neutrophils, lymphocytes, and occasional multinucleated giant cells. Additionally, hypertrophy of the airway secretory cells with an increase in mucin secretory matter was observed in animals exposed to OVA and concentrations of PFOA ranging from 0.5 to 1.5%. Sloughing of the epithelium and secretory cell necrosis was observed, with cells exhibiting condensed nuclei and nuclear debris (Fig. 5). This response was absent in the acetone control and PFOA alone–exposed animals, and no differences were observed between these groups (data not shown).

DISCUSSION

An increase in the incidence of asthma and allergic disease has been documented in urban areas and developed countries in the past 30–40 years (Von Hertzen and Haahtela, 2004). Although disease may be better diagnosed in these areas, the hygiene hypothesis provides one explanation for the apparent increase in hypersensitivity diseases, autoimmune disorders, and asthma. According to the hypothesis, advances in medicine (widespread use of vaccines early in childhood and the increased use of antibiotics) and improved living conditions (reduced family size and improved hygiene) have led to a dramatic decrease in childhood infections, reducing the

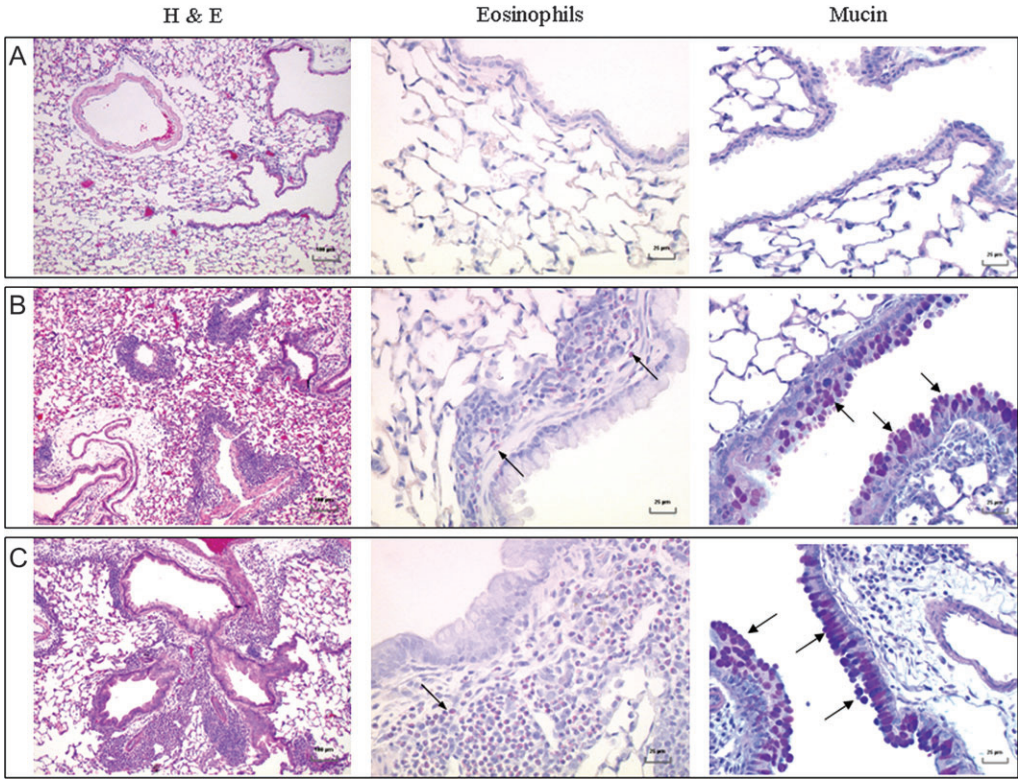

FIG. 5. Lung histopathology following exposure to OVA and increasing concentrations of PFOA. Photomicrographs represent (A) acetone control (PFOA, OVA), (B) OVA (IP and pulmonary challenged), and (C) OVA (IP and pulmonary challenged) and 1.0% PFOA-coexposed groups. Columns of slides are stained with H&E (pleiotropic response), modified Hansel's (eosinophilic response, with arrows pointing to the pink stained eosinophils), and Alcian Blue/PAS stain (arrows pointing to the dark purple stain indicate mucin). Each section is from one representative animal per dose group.

stimulation of the Th1 branch of the immune system (Rautava *et al.*, 2004; Schaub *et al.*, 2006; Von Hertzen and Haahtela, 2004; Yazdanbakhsh *et al.*, 2002). These findings have been supported by animal studies which have demonstrated that coexposure to endotoxin with allergen reduces the production of allergen-specific IgE and allergen-induced airway hyperreactivity (Howell *et al.*, 2004).

In concert with improved hygiene and reduced exposure to pathogens, individuals living in industrial society have increased exposures to chemicals. Although a wealth of literature exists demonstrating the immunosuppressive nature of certain chemicals, little has been done to investigate the impact of chemical-induced immunosuppression on an individual's ability to evoke an allergic response. Prior or concurrent xenobiotic exposure may alter or prime the immune system's response to antigens, skewing the Th1/Th2 balance. For example, exposure to diesel exhaust particles (DEP) has been shown to enhance allergen-induced Th2 and IgE responses (Dong *et al.*, 2005) but has also been shown to suppress the IgM response to sheep red blood cells (Yang *et al.*, 2002). Additionally, it has been demonstrated that challenge with DEP can alter the normal Th1 response to keyhole limpet hemocyanin (KLH) to a Th2 response with the production of KLH-specific IgE (as reviewed by Peden, 2002).

The toxicity data presented here agree with dermal and oral PFOA toxicity studies in rabbit, rat, and primate models. For example, Kennedy demonstrated an increase in liver weight without a decrease in body weights following dermal treatment of rabbits with 20 mg/kg of a related chemical, ammonium perfluorooctanoate (APFO) (Kennedy, 1985). Likewise, an increase in liver weight was demonstrated in rats following inhalation exposure (Kennedy *et al.*, 1986) or fed APFO (Griffith and Long, 1980) or PFOA (Butenhoff *et al.*, 2004b). The F1 generation of male rats had decreased body weights and concurrent increase in liver weights when both parents were exposed orally with 1–30 mg/kg APFO (Butenhoff *et al.*, 2004a). Rhesus monkeys fed 30 mg/kg/day APFO exhibited lipid depletion of the adrenals, hypocellularity of the bone marrow, and atrophy of the spleen and lymph nodes with no loss of body weight (Griffith and Long, 1980).

Additionally, these studies demonstrate an augmentation of the IgE and AHR to OVA when animals were concurrently exposed to OVA and PFOA. Our results are consistent with the results presented by Wilder *et al.* (1999) in the murine model of allergic asthma. OVA exposure in mice was characterized by increases in AHR after challenge with MCH, increased in OVA-specific IgE levels and eosinophil recruitment to the

lungs. Results from this study show similar results in animals exposed to OVA alone with an enhanced response seen in animals exposed to OVA and increasing concentrations of PFOA. Several mechanisms could be involved. Regulatory T cells have been shown to play a critical role in controlling asthma and allergy (Akbari *et al.*, 2003a) and may play a role in the augmentation of the IgE response to OVA following exposure to PFOA. PFOA has a high affinity (90% of administered dose) to bind to serum proteins in rat and human blood (Han *et al.*, 2003), and tissue distribution is primarily to the plasma and liver (Kudo and Kawashima, 2003). The liver has the highest concentration of any organ of natural killer (NK) T cells, a major type of CD4+ regulatory T cell. Invariant NK T cells capable of producing high levels of (interleukin) IL-4 and IL-13 have been identified in the lungs of asthmatic individuals (Akbari *et al.*, 2003a,b).

While NK T cells have been linked to asthma, a mechanism for their activation has not been well defined. As a peroxisome proliferator, PFOA is able to induce peroxisome proliferator–activated receptors (PPARs). PPARs are members of the nuclear hormone receptor superfamily and exist in three forms, α, β, and γ. Cells of the monocytes/macrophage lineage express both PPAR α and γ, suggesting a role for both of these receptors in immune function (Braissant *et al.*, 1996). Although recent literature demonstrates that activators of PPAR γ have been found to play a protective role in the pathogenesis of the toluene diisocyanate–induced asthma phenotype (Lee *et al.*, 2006), PFOA has been shown to activate PPAR α and β but not γ (Takacs and Abbott, 2006). Other PPAR α agonists, including, gemfibrozil, ciprofibrate, and fenofibrate, have been shown to indirectly increase the production of IL-4 (Lovett-Racke *et al.*, 2004) with gemfibrozil causing a shift in cytokine levels by inhibiting interferon-γ and promote IL-4 in human T-cell lines. This suggests the potential for PFOA to augment the Th2 response and subsequent airway hyperreactivity to OVA through a PPAR mechanism.

Epidemiological studies have detected elevated serum PFOA levels in community residents, and while no toxicity was noted with the limited end points evaluated, the need for further evaluations has been recognized (Emmett *et al.*, 2006b). Results of the studies presented here generated using a murine model of asthma have demonstrated that dermal exposure to PFOA, along with suppressing the Th1 arm of the immune system as demonstrated by Yang *et al.* (2002), has the potential to enhance IgE-mediated hypersensitivity. The persistence of PFOA in the environment in combination with these findings suggest that exposure to PFOA, although not allergenic itself, may enhance an individual's response to commonly encountered environmental allergens.

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