Toxicology 267 (2010) 132–139

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

28-Day dietary exposure of mice to a low total dose (7 mg/kg) of perfluorooctanesulfonate (PFOS) alters neither the cellular compositions of the thymus and spleen nor humoral immune responses: Does the route of administration play a pivotal role in PFOS-induced immunotoxicity?

Mousumi Rahman Qazi, B. Dean Nelson, Joseph W. DePierre, Manuchehr Abedi-Valugerdi*

Department of Biochemistry and Biophysics, Arrhenius Laboratories for the Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 10 September 2009
Received in revised form 30 October 2009
Accepted 30 October 2009
Available online 10 November 2009

Keywords:
Humoral immune responses
T-cell-independent antigen
T-cell-dependent antigen
Perfluorooctanesulfonate (PFOS)

ABSTRACT

Short-term exposure of mice to high doses of perfluorooctanesulfonate (PFOS), an ubiquitous and highly persistent environmental contaminant, induces various metabolic changes and toxic effects, including immunotoxicity. However, extrapolation of these findings to the long-term, low-dose exposures to which humans are subject is highly problematic. In this connection, recent studies have concluded that sub-chronic (28-day) exposure of mice by oral gavage to doses of PFOS that result in serum levels comparable to those found in general human populations suppress adaptive immunity. Because of the potential impact of these findings on environmental research and monitoring, we have examined here whether sub-chronic dietary exposure (a major route of human exposure) to a similarly low-dose of PFOS also suppress adaptive immune responses. Dietary treatment of male B6C3F1 mice for 28 days with a dose of PFOS that resulted in a serum concentration of 11 μg/ml (ppm) significantly reduced body weight gain and increased liver mass. However, this treatment did not alter the cellular compositions of the thymus and spleen; the number of splenic cells secreting IgM antibodies against sheep red blood cell (SRBC); serum levels of IgM and IgG antibodies specifically towards SRBC; or circulating levels of IgM antibodies against the T-cell-independent antigen trinitrophenyl conjugated to lipopolysaccharide (TNP–LPS). These findings indicate that such sub-chronic dietary exposure of mice to PFOS resulting in serum levels approximately 8–85-fold greater than those observed in occupationally exposed individuals does not exert adverse effects on adaptive immunity.

1. Introduction

The unique chemical properties of perfluorooctanesulfonate (PFOS; $C_8F_{17}SO_3^-$), including potent surfactant activity, chemical inertness and exceptional stability to both metabolic and environmental degradation, have been exploited in numerous industrial and consumer applications (Kissa, 1985; Lehmler, 2005; Lau et al., 2007), while, at the same time, creating environmental concerns. In large part because of the ubiquitous distribution of this fluorochemical in the general environment (Butenhoff et al., 2006; Houde et al., 2006; Lau et al., 2007) and its long elimination half-life in humans (several years) (Olsen et al., 2007), research interest in its potential toxicity towards living organisms is extensive and still growing (U.S. EPA, 1998; OECD, 2002; 3M Company, 2003), and the toxic effects exerted by PFOS on experimental animals have been reviewed (3M Company, 2003; Lau et al., 2004, 2007).

Short-term, high-dose exposure of rodents to PFOS has numerous effects, including pronounced hepatomegaly and peroxisome proliferation; reduced appetite and loss of body weight and fat; changes in hormonal status; reproductive toxicity; as well as, following long-term exposure, tumors of the liver. Moreover, it has been demonstrated in our laboratory and others that PFOS can suppress various aspects of adaptive immunity (Lau et al., 2007; Keil et al., 2008; DeWitt et al., 2009; Dong et al., 2009; Qazi et al., 2009a; Zheng et al., 2009), while activating the innate immune system of mice (Qazi et al., 2009b). At sufficiently high sub-chronic doses, PFOS also disrupts carbohydrate and lipid metabolism in rodents and monkeys (Ikeda et al., 1985; Haughom and Spydevold, 1992; Seacat et al., 2002, 2003; Luebker et al., 2005; Curran et al., 2008).

Most of these studies have involved short-term, high-dose exposure to PFOS and, as is always the case in such a situation, extrapolation of the findings to more realistic long-term, low-dose

Abbreviations: PFOS, perfluorooctanesulfonate; TNP–LPS, trinitrophenyl–lipopolysaccharide; SRBC, sheep red blood cells.

* Corresponding author. Tel.: +46 8 164240; fax: +46 8 153679.
E-mail address: abedi@dbb.su.se (M. Abedi-Valugerdi).

0300-483X/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2009.10.035

exposure is highly problematic. In this context, Peden-Adams et al. (2008) reported recently that 28-day exposure of male B6C3F1 mice to PFOS via oral gavage at levels that produce serum concentrations similar to those detected in general human populations (i.e., 0.05–5 mg PFOS/kg total administered dose (TAD), which produced serum concentrations of approximately 0.09–0.666 μg/ml) suppress both T-cell-dependent and -independent IgM antibody responses. On the other hand, in another recent study, Dong et al. (2009) found that 60-day exposure of male C57BL/6 mice by this same route and mode of administration to a TAD of 0.5 mg PFOS/kg, resulting in mean serum levels of 0.67 μg/ml, has no effect on at least certain aspects of the adaptive immune system. These latter investigators also found that at TAD of 5–125 mg/kg (yielding serum concentrations of 0.674–121 μg/ml) PFOS can suppress specific humoral immune responses.

Consumption of contaminated food has been identified as a significant source of PFOS for general populations in Western countries (Fromme et al., 2009). The United Kingdom (UK) Food Standards Agency has recently estimated that in that country the upper-bound average dietary exposure to PFOS is 0.010 μg/kg body weight/day, with a 97.5th percentile intake of 0.02 μg/kg body weight/day (http://www.food.gov.uk/multimedia/pdfs/fsis0509.pdf). Estimates of daily dietary intake of PFOS in Canada (Tittlemier et al., 2007), Germany (Fromme et al., 2007), and Spain (Ericson et al., 2008) range from 0.0006 to 0.0044 μg/kg body weight.

Therefore, the present investigation was designed to determine whether sub-chronic dietary exposure to low levels of PFOS produces immunotoxic effects in mice. Mice were administered approximately 250 μg PFOS/kg body weight in their diet for 28 days in order to obtain a final serum concentration that was reasonably realistic, i.e., similar to the highest serum concentrations reported in individuals exposed occupationally. The parameters monitored were the cellular compositions of the thymus and spleen and the ability of the exposed animals to produce antibodies against both T-cell-dependent and -independent antigens. Our present findings suggest that, unlike what has been reported in the case of exposure by oral gavage, 28-day dietary exposure of mice to an environmentally relevant dose of PFOS does not compromise humoral immune responses.

2. Materials and methods

2.1. Animals

Male B6C3F1 (H-$2^{b/k}$) mice (7–8 weeks old at the beginning of each experiment) were purchased from Taconic (Taconic Europe A/S, Ry, Denmark). Male mice were chosen to avoid the hormonal changes associated with cyclic ovulation and menstruation, which can influence the immune system (Verthelyi, 2001; Bouman et al., 2005). Moreover, Peden-Adams et al. (2008) demonstrated that male B6C3F1 mice are more sensitive than females of the same strain to the immunotoxicity of low doses of PFOS. These animals were housed at the Wenner-Gren Institute, Stockholm University, at 22 °C with a 12-h light/12-h dark cycle, 50% humidity and access to the diets indicated below and tap water *ad libitum*. Upon arrival, groups of five mice each were placed in separate cages (for group monitoring of food intake) and allowed to acclimatize to this environment for at least 7 days before initiating dietary exposure to PFOS. These experiments were pre-approved by the Northern Stockholm Ethical Committee for Animal Experimentation (approval number N150/07).

2.2. Preparation of the diet

An aqueous solution of PFOS (the tetraethylammonium salt; FW: 629.4; purity >98%, Sigma–Aldrich Sweden AB, Stockholm, Sweden) was blended thoroughly with powdered RMI (E) FG SQC basal diet (containing 2.71% fat, 14.38% protein and 61.73% carbohydrate; SDS, Special Diets Services, Essex, UK) to obtain a homogeneous mixture containing 0.000156% (1.56 μg/g, w/w) of this fluorochemical. This mixture was then dried for 48 h in a hood (in order to obtain cakes of chow, which allow much more accurate assessment of food consumption than with a powdered diet, which mice scatter all over their cages) and subsequently stored at 4 °C prior to use. Since these mice weighed roughly 25 g and consumed about 4 g of chow daily, their intake was approximately 250 μg PFOS/kg body weight/day or 7 mg/kg for the entire 28-day experimental period. The control diet was prepared in the same manner, except that no PFOS was added.

2.3. Experimental design

For practical reasons, three separate studies were conducted, all involving five mice in each individual experimental group. In **Study 1**, two groups were given access to either control or PFOS-containing diet *ad libitum* for 28 consecutive days. During this period, the animals were weighed weekly and their food intake determined by subtracting the weight of the food remaining at the end of each 1-week period from the initial weight of the chow provided. At the end of the treatment period the thymus, spleen, liver and epididymal fat were dissected out and weighed. Organ mass was normalized for body weight and is reported as a somatic index (i.e., [organ weight (g)/body weight (g)] × 100).

In **Study 2**, two groups received the PFOS-containing and two others control diet, again for 28 days. On day 23 (5 days prior to euthanasia), the animals in one treated and one control group were injected intraperitoneally with 0.1 ml of a suspension of packed sheep red blood cells (SRBC, SVA, Uppsala, Sweden) diluted 1:10 in phosphate-buffered saline (PBS) in order to examine T-cell-dependent humoral immune responses (Harper et al., 1993). Following sacrifice on day 28, serum levels of immunoglobulins (total IgG and IgM) directed specifically against the sheep red blood cells were assayed, the total number of plasma cells in the spleen determined, and the haemagglutination and plaque-forming cell (PFC) assays performed (see below for further details).

Study 3 also involved four groups of mice, two of which received PFOS-containing and the other two control diets for 28 days. In order to evaluate responses to a T-cell-independent antigen, on day 23 the animals in one treated and one control group were injected intravenously through the tail vein with 0.1 ml trinitrophenyl conjugated to lipopolysaccharide (100 μg/ml) (Sigma–Aldrich Sweden AB, Stockholm, Sweden) dissolved in 0.9% NaCl. Following sacrifice 5 days later, serum levels of IgM specific for this antigen were assayed (see below).

2.4. Collection and processing of blood samples, thymuses and spleens

At the end of the period of feeding, all mice in all three studies were bled by retroorbital puncture under light isoflurane anesthesia and thereafter killed by cervical dislocation. The blood samples were collected in 0.5 ml Vacutainer serum separator tubes (Microtainer BD Bioscience, NJ, USA), allowed to clot at 4 °C and then centrifuged at 1300 × $g_{av.}$ The sera thus obtained were stored in aliquots at −80 °C until being analyzed for their concentrations of PFOS, capacities for haemagglutination or levels of immunoglobulins.

The spleens and thymuses were suspended in PBS and teased apart into single-cell suspensions with a tissue forceps and the cells thus collected were washed twice in PBS. In order to lyse the erythrocytes present, the cells were pelleted by centrifugation, incubated with 2 ml of an ammonium chloride-based lysing buffer and then re-pelleted. The resulting pellets were then re-suspended in 0.5 ml PBS containing 1% fetal calf serum (FCS) and 0.1% NaN_3 (hereafter referred to as FACS buffer) for flow cytometric analysis of their cell-surface phenotypes (see below). These same suspensions were employed to count the number of thymocytes and splenocytes using a haemocytometer and to assess their viability on the basis of trypan blue exclusion. This viability was routinely greater than 90%.

2.5. Determination of serum levels of PFOS by HPLC–MS/MS

For this analysis, PFOS-containing two O^{18} atoms in the sulfonate group (99% purity, synthesized by Research Triangle Institute, Research Triangle Park, NC) was used as an internal standard and added in varying amounts to blank matrix and all of the samples in order to obtain appropriate internal standard curves. On the basis of their expected PFOS concentrations (as judged from previous experience), the serum samples from exposed and control mice were diluted 50- and 5-fold, respectively, prior to analysis. All dilutions were performed with heat-inactivated fetal calf serum (FCS) in new, disposable polypropylene tubes.

100-μl aliquots of these diluted serum samples containing the internal standard were then extracted using a solid-phase technique (Kuklenyik et al., 2004; Ehresman et al., 2007). In brief, 1.0 ml formic acid and 100 μl saturated ammonium sulfate were added to each sample and the resulting mixture vortexed prior to extraction with Oasis® hydrophilic–lipophilic balanced 3.0-ml cartridges (Waters, Milford, MA, USA).

Prior to quantitation by mass spectrometry, the PFOS in each sample was separated on a Mac-Mod Analytical HPLC column (ACE C-18, 5 μm, 100 × 2.1 mm i.d.; Mac-Mod, Chadds Ford, PA, USA). The mobile phase of 51% acetonitrile and 49% 2 mM ammonium acetate was applied at an isocratic flow rate of 0.25 ml/min. The fraction containing PFOS was subsequently analyzed on an API 4000 mass spectrometer (Applied Biosystems/MDS-Sciex Instrument Corporation, Forest City, CA) equipped with a pneumatic electrospray ionization source (Turbo Ion Spray) and run in the negative ion mode. The negative parent ions and transition ions for the PFOS (499–80 atomic mass units (amu), respectively) and the internal standard (503–84 amu, respectively) were monitored and the PFOS content of the samples determined by comparison to the standard curve. All of the parameters involved in this analytical system were optimized for use with mouse serum.

2.6. Measurement of serum levels of IgM and IgG antibodies specific for sheep red blood cells (SRBC)

Serum levels of IgM and IgG antibodies specifically against SRBC were measured with an enzyme-linked immunosorbent assay (ELISA) procedure as described by Heyman et al. (1984) with slight modifications. In brief, these cells were washed three times with PBS, packed by centrifugation and diluted 1:1000 in this same buffer, following which 150 μl of this suspension was added to each well of 96-well U-bottomed plates (BD Labware, NJ, USA). After the cells had been allowed to settle for 1 h at room temperature, the plates were immersed in 0.25% glutaraldehyde dissolved in PBS for 5 min at room temperature, in order to fix the cells to the surface of the wells.

After three subsequent washes with PBS containing 0.2% Tween, 150 μl 1% bovine serum albumin (BSA) dissolved in PBS was added to each well to block free binding sites, followed by incubation for 2 h at 37 °C. Thereafter, the plates were again washed three times and 50 μl of sera diluted serially with PBS-1% BSA (starting with a dilution of 1:100) were added to different wells and incubation continued overnight at 4 °C. After three more washes, 50 μl goat anti-mouse IgM or IgG conjugated with alkaline phosphatase was added (at a dilution of 1:2000; Southern Biotechnology) to each well, followed by incubation for 2 more hours at 37 °C and three more washes. Finally, the plates were incubated for 30 min at room temperature in a solution of *p*-nitrophenyl phosphate (disodium salt hexahydrate; Sigma), the phosphatase substrate, and the absorbance of the wells at 405 nm determined on a microplate reader (SpectraMAX 250).

2.7. Measurement of serum levels of IgM antibodies specific for the hapten trinitrophenyl (TNP)

Serum levels of IgM antibodies directed specifically against the hapten TNP were determined utilizing an ELISA procedure. In brief, individual wells on 96-well flat-bottomed plates (Nunc A/S, Roskilde, Denmark) were coated with 100 μl TNP (10 μg/ml) conjugated to bovine gamma globulin (TNP–BGG) (BioCat GmbH, Heidelberg, Germany) dissolved in PBS by incubation overnight at 4 °C, after which remaining free binding sites were blocked by incubation with 1% bovine serum albumin (BSA) dissolved in PBS for 2 h at room temperature. After washing three times with PBS–Tween, 100 μl mouse serum diluted 50–800-fold with PBS–albumin was added to individual wells, followed by incubation at 37 °C for 3 h. Following three more washes with PBS–Tween, 0.1 ml goat anti-mouse IgM conjugated with alkaline phosphatase (diluted 1:2000; Southern Biotechnology) was added to each well, incubation continued for 2 h at room temperature and five more washes were then performed. Finally, hydrolysis of the substrate *p*-nitrophenyl phosphate was monitored at 405 nm as described above.

2.8. Measurement of serum levels of corticosterone

The levels of corticosterone in serum samples were quantitated employing a commercially available ELISA kit (Assay Designs, MI, USA) in accordance with the manufacturer's instruction. In all cases, quantitative values were obtained by comparison with the standard curve obtained with corticosterone provided by the manufacturer.

2.9. The plaque-forming assay

For this assay, a single-cell suspension of splenocytes was obtained by gentle disruption of the organ in Earle's balanced salt solution (EBSS; GIBCO) with a tissue forceps, following which the cells were washed three times and then resuspended in EBSS. Thereafter, the number of spleen cells secreting IgM antibodies directed specifically against SRBC were determined employing the Cunningham plaque assay (Cunningham and Szenberg, 1968). In brief, 25 μl of each spleen cell suspension was mixed with 25 μl packed sheep red blood cells (diluted 1:20 in BSS containing 5% (v/v) guinea pig complement reconstituted in accordance with the manufacturer Cederlaneïs instructions) and this mixture then transferred to a Cunningham–Szenberg chamber slide. The slide was sealed with melted paraffin wax and incubation subsequently performed for 1 h at 37 °C. The plaques formed were counted under a magnifying glass and the results expressed as the number of plaque-forming cells (PFC) per million cells.

2.10. The haemagglutination assay

Agglutination of SRBC by the sera of both immunized and non-immunized mice was determined as described by Hudson and Hay (1989). In brief, the serum samples were diluted serially from 5- to 320-fold in PBS and 25 μl of each such dilution mixed with 25 μl packed SRBC (diluted 1:10 in PBS) in the individual wells of V-bottomed microtitre plates (BD Labware, NJ, USA). These plates were subsequently incubated at room temperature for 1 h and the most diluted sample that resulted in visible haemagglutination recorded as the titer.

2.11. Flow cytometric phenotyping of thymocytes and splenocytes

The phenotypically distinct subpopulations of cells present in the spleen and thymus were analyzed by first staining these cells with antibodies directed against the appropriate surface antigen markers and then detecting stained cells by flow cytometry in a BD FACS Calibration System (San Jose, CA, USA). The following monoclonal antibodies directed against the specific murine cell-surface antigens indicated and labeled with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE) or peridinin chlorophyll protein (PerCP) (all purchased from BD PharMingen, SanDiego, CA) were employed: CD19-FITC (clone 1D3), γδ T-cell receptor-FITC (clone GL3), CD11b-FITC (clone M1/70), CD4-FITC (clone RM4-4 H57-597), CD49b/Pan-NK-PE (clone DX5), Gr1-PE (clone H57-597), CD138-PE (281-2) and CD8a-PerCP (clone 53-6.7). Single staining was employed for the detection of B cells ($CD19^+$), plasma cells ($CD138^+$) and γδ T cells ($\gamma\delta\ T^+$) and double staining in the cases of CD4/CD8 ($CD4^+/CD8^+$), NK/NKT ($CD49b^+/CD4^+$) and macrophage/neutrophil ($CD11b^+/Gr1^+$) subpopulations among thymic and splenic cells. Unstained cells, as well as rat IgG conjugated with FITC or PE were used to establish gates for the cells expressing specific surface marker(s). Dead cells were excluded on the basis of their forward and side scatter.

For each analysis, 10^6 thymocytes or splenocytes were mixed with the appropriate antibody solution in 50 μl FACS buffer and thereafter incubated on ice for 30 min in the dark, in accordance with the manufacturer's instructions. The signals emitted by FITC, PE and PerCP were detected at 530, 575 and 670 nm, respectively. For each sample, data from 10,000 events (individual cells) were collected and analyzed with the CellQuest Software. The sizes of the various cell populations are expressed both in terms of absolute numbers and percentages of the total number of cells present.

2.12. Statistical analysis

All data are expressed as means ± standard errors (SE) and differences between the values for the control and exposed groups examined for statistical significance by the Mann–Whitney (*U*-test) at the significance level $2\alpha = 0.05$ as indicated in the tables and legends to the figures. All statistical analyses were performed utilizing the WinSTAT software (R. Fitch Software, Medina AB, Vänerborg, Sweden).

3. Results

3.1. Effects of sub-chronic, low-dose dietary exposure of mice to PFOS on food intake, body and organ mass, and serum levels of corticosterone

Male B6C3F1 mice were exposed here via their diet to a TAD of 7 mg PFOS tetraethylammonium salt/kg body weight (which is equivalent to a TAD of 5.55 mg PFOS anion/kg) during a period of 28 days. This dose is approximately the same as the maximal TAD applied routinely by Peden-Adams et al. (2008), also over a 28-day period (5 mg), as well as the lowest TAD reported by Dong et al. (2009) to suppress humoral immune responses in mice following

Table 1
The effects of sub-chronic (28-day) dietary exposure of a low-dose (5.55 mg/kg TAD) of PFOS on body weight gain; the relative masses of the liver, thymus, spleen and epididymal fat pads; and serum corticosterone in male B6C3F1 mice.[a]

Exposure	Body weight gain (g)[b]	Liver mass[c]	Thymus mass[c]	Spleen mass[c]	Fat mass[c,d]	Serum corticosterone (ng/ml)
None	4.2 ± 0.45	4.8 ± 0.14	0.094 ± 0.002	0.23 ± 0.007	2.2 ± 0.24	37.5 ± 3.1
PFOS	3.3 ± 0.13[e]	5.3 ± 0.12[e]	0.084 ± 0.006	0.27 ± 0.015	1.6 ± 0.25[*]	47.5 ± 4.8[*]

[a] All values are means ± SE (standard error) for five animals in each group. Differences were analyzed statistically employing the Mann–Whitney *U*-test.
[b] Change in body weight from day 0 to day 28 of exposure.
[c] Calculated as [organ weight (g)/body weight (g)] × 100.
[d] Epididymal fat pads.
[e] Statistically significant compared with the control group.
[*] $P \geq 0.06$.

Table 2
Effects of 28-day dietary treatment of male B6C3F1 mice with 0.000156% (w/w) perfluorooctanesulfonate (PFOS) on the numbers and proportions of phenotypical distinct subpopulations of thymocytes.[a].

Cell phenotype (specific cell-surface marker)	Control mice		PFOS-treated mice		
	Number ($\times 10^6$)	% of total	Number ($\times 10^6$)	% of total	% of control
Cells of all phenotypes	56.1 ± 6.0[b]	100	47.8 ± 7.0	100	(85)
Immature cells ($CD4^-CD8^-$)	1.7 ± 0.3	3.1 ± 0.2	1.6 ± 0.4	3.3 ± 0.2	(94)
Immature cells ($CD4^+CD8^+$)	47.1 ± 9.5	84.0 ± 2.0	39.5 ± 11.4	82.2 ± 1.0	(84)
Mature T helper cells ($CD4^+CD8^-$)	5.2 ± 1.4	9.2 ± 1.2	4.8 ± 1.1	10.3 ± 0.7	(92)
Mature T cytotoxic cells ($CD8^+CD4^-$)	1.4 ± 0.4	2.4 ± 0.4	1.3 ± 0.3	2.8 ± 0.3	(92)
Natural killer T cells ($CD4^+$Pan-NK^+)	0.5 ± 0.2	1.0 ± 0.3	0.5 ± 0.1	1.0 ± 0.1	(100)
Natural killer cells ($CD4^-$Pan-NK^+)	0.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	(100)

[a] Male B6C3F1 mice, five in each group, received diets containing no added xenobiotic (the control group) or 0.000156% (w/w) PFOS for 28 days. At the end of this period, the animals were sacrificed by cervical dislocation and their thymuses were removed by dissection. Suspensions of individual cells were then prepared from these organs by mechanical disruption with a tissue forceps and the total cell numbers counted, examined for viability (on the basis of Trypan blue exclusion) and phenotyped (by staining with appropriate monoclonal antibodies, followed by flow cytometric analysis).
[b] All values presented are means ± SE (standard error). No statistically significant differences were observed.

60 days of exposure. This regimen of exposure was tolerated well by our animals, e.g., the PFOS-treated and control mice consumed an identical amount of chow (4.1 g/day versus 4.2 g/day) and exposure did not significantly alter serum levels of corticosterone, an indicator of stress (Table 1). Nonetheless, at the end of the treatment period, the exposed mice had gained somewhat less weight than the untreated controls (Table 1).

As also shown in Table 1, following this 28-day dietary treatment the liver mass was elevated significantly, whereas the masses of the thymus, spleen and epididymal fat pads were not significantly different than the corresponding control values.

3.2. Serum levels of PFOS following sub-chronic, low-dose dietary exposure

Following 28 days of dietary exposure to a TAD of 5.55 mg of the PFOS anion/kg body weight, the level of this compound in the serum of our mice was found to be 11.6 ± 0.2 μg/ml (ppm), as compared to 0.0409 ± 0.0023 μg/ml (ppm) in the unexposed animals ($n = 5$ in both cases).

3.3. Effects of sub-chronic, low-dose dietary exposure to PFOS on the absolute and relative sizes of phenotypically distinct subpopulations of cells in the thymus and spleen

This dietary exposure had no effect on the total numbers of circulating leukocytes (not shown) or of cells in the thymus (Table 2) and spleen (Table 3) of male B6C3F1 mice. Nor did this treatment alter the relative numbers of the phenotypically distinct types of cells present in the thymus (i.e., immature $CD4^-/CD8^-$ and $CD4^+/CD8^+$ lymphocytes, mature T helper cells, mature T cytotoxic cells, natural killer T cells and natural killer cells) (Table 2) or spleen (i.e., B lymphocytes, T helper cells, T cytotoxic cells, Tγδ cells, natural killer T (NKT) cells, natural killer (NK) cells, macrophages, granulocytes and plasma cells) (Table 3).

3.4. Effects of sub-chronic, low-dose dietary exposure to PFOS on T-cell-dependent humoral immune responses

In order to determine if our sub-chronic dietary exposure of mice to a relatively low-dose of PFOS affects humoral antibody responses to a T-cell-dependent antigen, PFOS-treated and control mice were immunized with SRBC on day 23 of the treatment period and antibody responses to this antigen monitored 5 days later, on the final day of treatment, employing four different approaches. First, we determined the proportion of plasma ($CD138^+$) cells in the spleen, as well as the number of these cells that were secreting anti-SRBC IgM antibodies employing flow cytometry and the plaque-forming assay, respectively. As shown in Fig. 1a and b, there were no differences between the control and exposed groups with respect to these immune parameters. In order to confirm this finding by determining serum levels of anti-SRBC antibodies, we performed a haemagglutination assay utilizing SRBC as the target cells. As documented in Fig. 2, a titer of approximately 1:80 was obtained for both the exposed and unexposed mice.

To be even more certain of these humoral responses of PFOS-treated and untreated mice to immunization with SRBC, we employed ELISA assays to measure their circulating levels of both

Table 3
Effects of 28-day dietary treatment of male B6C3F1 mice with 0.000156% (w/w) perfluorooctanesulfonate (PFOS) on the numbers and proportions of phenotypical distinct subpopulations of splenocytes.[a].

Cell phenotype (specific cell-surface marker)	Control mice		PFOS-treated mice		
	Number ($\times 10^6$)	% of total	Number ($\times 10^6$)	% of total	% of control
Cells of all phenotypes	62.2 ± 8.0[b]	100	70.7 ± 4.6	100	(113)
B cells ($CD19^+$)	28.5 ± 4.4	45.2 ± 2.5	32.7 ± 2.5	46.2 ± 1.6	(114)
T helper cells ($CD4^+$)	12.7 ± 1.4	20.7 ± 0.9	13.7 ± 0.8	19.5 ± 0.8	(107)
T cytotoxic cells ($CD8^+$)	7.3 ± 0.8	11.7 ± 0.6	7.9 ± 0.5	11.2 ± 0.2	(108)
T γδ cells ($\gamma\delta^+$)	2.2 ± 0.3	3.5 ± 0.3	2.4 ± 0.2	3.4 ± 0.3	(109)
Natural killer T ($CD4^+$Pan-NK^+)	0.7 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	(114)
Natural killer ($CD4^-$Pan-NK^+)	3.5 ± 0.4	5.6 ± 0.5	4.1 ± 0.3	5.9 ± 0.5	(114)
Macrophages ($CD11b^+$)	3.8 ± 0.4	6.2 ± 0.7	4.9 ± 0.5	7.0 ± 0.9	(128)
Granulocytes ($CD11b^-Gr/1^+$)	3.1 ± 0.4	5.1 ± 0.4	3.5 ± 0.3	5.0 ± 0.4	(112)
Plasma cells ($CD138^+$)	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.03	0.8 ± 0.1	(125)

[a] Male B6C3F1 mice, five in each group, received diets containing no added xenobiotic (the control group) or 0.000156% (w/w) PFOS for 28 days. At the end of this period, the animals were sacrificed by cervical dislocation and their spleens were removed by dissection. Suspensions of individual cells were then prepared from these organs by mechanical disruption with a tissue forceps and the total cell numbers counted, examined for viability (on the basis of Trypan blue exclusion) and phenotyped (by staining with appropriate monoclonal antibodies, followed by flow cytometric analysis).
[b] All values presented are means ± SE (standard error). No statistically significant differences were observed.

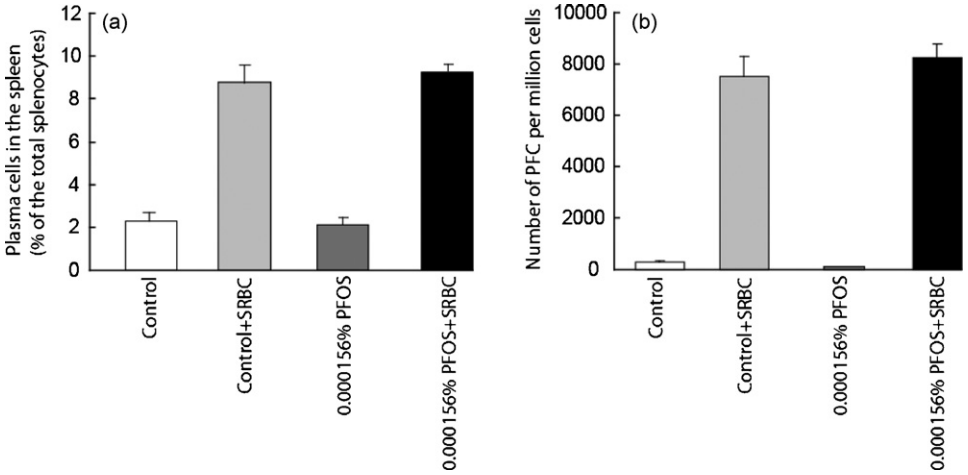

Fig. 1. (a) The relative proportion (percentage) of plasma cells and (b) the number of plaque-forming cells (PFC) in the spleen of mice immunized with sheep red blood cells (SRBC) during dietary exposure to 5.55 mg PFOS cation/kg (TAD) over a period of 28 days. Male B6C3F1 mice, five in each group, received diets containing no added xenobiotic (control) or 0.000156% (w/w) PFOS for 28 days. On day 23 of exposure, a suspension of sheep red blood cells was injected intraperitoneally into each mouse and when the animals were sacrificed 5 days later, their spleens were dissected out and teased into a single-cell suspension with a tissue forceps. Thereafter, the percentage of splenic plasma cells (i.e., antibody-producing lymphocytes) present was examined by staining with an appropriate monoclonal antibody and subsequent flow cytometric analysis (a) and the number of cells exhibiting a specific IgM response to SRBC (plaque-forming cells, PFC) was determined employing the plaque-forming assay and (b) (for further details, see Section 2). The values presented are means ± standard errors ($n = 5$). There were no statistically significant differences between the exposed and control animals, as determined by Mann–Whitney (U-test) analysis.

IgM and IgG antibodies directed specifically against these cells and, once again, found no differences between the two groups (Fig. 3a and b).

3.5. *Effects of sub-chronic, low-dose dietary exposure to PFOS on T-cell-independent humoral immune responses*

As illustrated in Fig. 4, we also found no effect of sub-chronic dietary exposure of mice to this relatively low-dose of PFOS on their IgM antibody response to the T-cell-independent antigen trinitrophenyl–lipopolysaccharide (TNP–LPS).

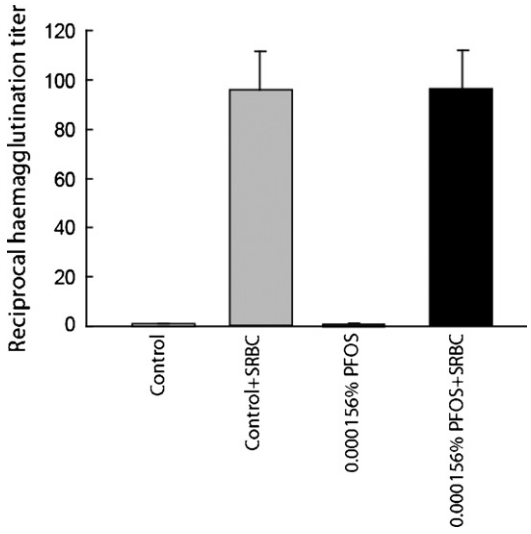

Fig. 2. Haemagglutination of sheep red blood cells (SRBC) by serial dilutions of sera from PFOS-treated and control mice with and without immunization by SRBC. Male B6C3F1 mice, five in each group, received diets containing no added xenobiotic (control) or 0.000156% (w/w) PFOS for 28 days. On day 23 of exposure, a suspension of sheep red blood cells was injected intraperitoneally into each mouse and when the animals were sacrificed 5 days later, blood samples were collected and serial dilutions of these samples analyzed employing a haemagglutination assay (for further details, see Section 2).

4. Discussion

Induction of immunotoxicity by perfluorinated compounds, including PFOS, is regarded by the U.S. Environmental Protection Agency as an end-point of concern in connection with risk assessment (U.S. EPA, 2006). This same agency suggests that such immunotoxicity in rodents should be evaluated in relationship to the serum concentration of the perfluorinated compound administered. In the present investigation exposure of adult male B6C3F1 to a TAD of 5.55 mg PFOS anion/kg body weight over a period of 28 days resulted in a serum concentration of 11.6 ± 0.2 μg/ml, which is 1–2 orders of magnitude higher than the levels observed in most occupationally exposed workers and approximately equal to the highest level reported in such workers (OECD, 2002).

Here, mice with this serum level of PFOS exhibited an increase in liver size, without any significant alteration in the masses of the thymus and spleen, both of which play key roles in adaptive immunity. These findings are consistent with those of Dong et al. (2009), who reported that following exposure of adult male C57BL/6 mice to a TAD of 5 mg PFOS/kg by gavage over a period of 60 days (which resulted in a serum concentration of 7.13 ± 1.03 μg/ml), the size of the liver is increased, while those of the thymus and spleen remain unaltered. On the other hand, Peden-Adams et al. (2008) found that exposure of adult male B6C3F1 mice to an identical dose of PFOS via gavage for 28 days did not affect the size of any of these three organs.

We also observed here that 28-day dietary exposure of male B6C3F1 mice to a TAD of 5.55 mg PFOS anion/kg body weight did not alter the cellular compositions of the thymus and spleen in any way. In line with these observations, Dong et al. (2009) found that treatment of male C57BL/6 mice with PFOS via gavage for 60 days only results in significant changes in the cellular compositions of the thymus and spleen at TAD of 25 mg/kg (resulting in a serum concentration 21.6 ± 4.4 μg PFOS/ml) and greater. In contrast, Peden-Adams et al. (2008) found a significant reduction in the population of $CD4^+$ T helper cells, together with an enhanced number of $CD8^+$ T cytotoxic cells in the spleen of male B6C3F1 mice upon exposure via oral gavage for 28 days to a TAD as low as 0.1 mg/kg (which yields a serum concentration 0.131 ± 0.015 μg/ml).

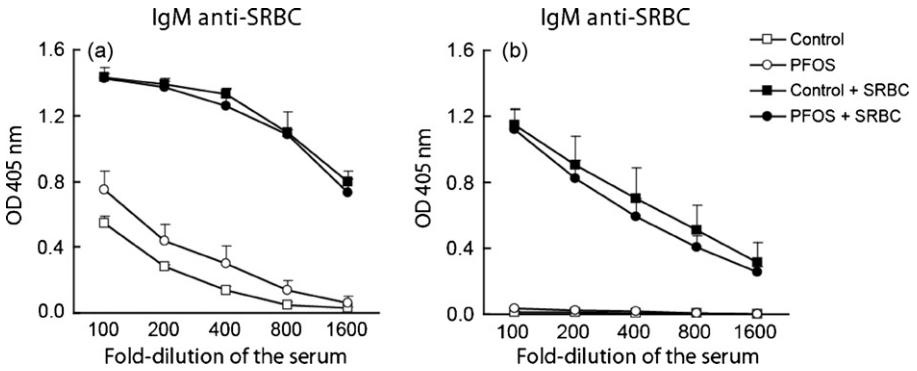

Fig. 3. Serum levels of specific IgM (a) and IgG (b) antibodies in mice immunized with sheep red blood cells (SRBC) during dietary exposure to 5.55 mg PFOS cation/kg (TAD) over a period of 28 days. Male B6C3F1 mice, five in each group, received diets containing no added xenobiotic (control) or 0.000156% (w/w) PFOS for 28 days. On day 23 of exposure, a suspension of sheep red blood cells was injected intraperitoneally into each mouse and when the animals were sacrificed 5 days later, blood samples were collected and serial dilutions of these samples analyzed for their levels of IgM and IgG antibodies directed specifically against these cells employing an ELISA procedure (for further details, see Section 2). The values presented are means ± standard errors ($n = 5$). There were no statistically significant differences between the exposed and control animals, as determined by Mann–Whitney (*U*-test) analysis.

One standard procedure for assessing the immunological status of mice is to determine the number of plasma cells in the spleen, which upon exposure to SRBC produce specific, T-cell-dependent IgM antibodies (U.S. EPA, 1998). Here, we observed no significant change in this parameter in our adult male B6C3F1 mice with our regimen of exposure. In order to confirm this finding of the unattenuated ability of our PFOS-treated mice to produce specific antibodies towards sheep red blood cells, we performed three additional determinations not reported earlier. First, we demonstrated that the number of $CD138^+$ plasma cells (i.e., cells actively producing IgM antibodies, as determined by flow cytometric analysis) in the spleen of the exposed animals challenged with sheep red blood cells was the same as in control mice. In addition, we showed that the circulating levels of specific IgM and IgG antibodies in these mice (as determined employing both an ELISA procedure and a haemagglutination assay) were the same as in unexposed animals.

Together, these findings provide strong support for our conclusion that a TAD of as much as 5.55 mg PFOS anion/kg body weight administered in the diet over a period of 28 days does not reduce the ability of adult male B6C3F1 mice to produce antibodies towards the T-cell-dependent antigen, SRBC. Moreover, our finding that in response to a challenge with TNP–LPS, PFOS-treated and untreated male B6C3F1 mice produce essentially identical serum levels of specific IgM antibodies indicate that T-cell-independent humoral responses also remain unaltered following the regimen of exposure employed here. In contrast to these present findings concerning functional aspects of the adaptive immune system, Peden-Adams et al. (2008) and Dong et al. (2009) report that 28- or 60-day exposure of either adult male B6C3F1 or C57BL/6 mice, respectively, to a TAD of 5 mg PFOS/kg via oral gavage substantially suppress humoral antibody responses to both T-cell-dependent and -independent antigens.

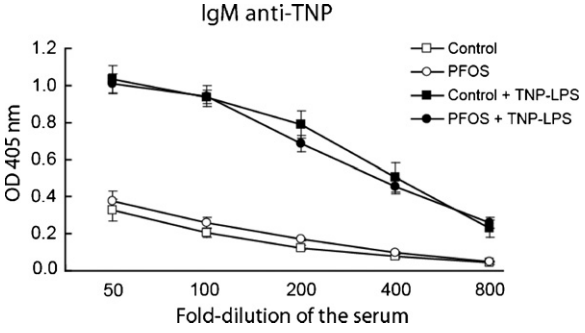

Fig. 4. Serum levels of specific IgM antibodies in mice immunized with trinitrophenyl–lipopolysaccharide (TNP–LPS) during dietary exposure to 5.55 mg PFOS cation/kg (TAD) over a period of 28 days. Male B6C3F1 mice, five in each group, received diets containing no added xenobiotic (control) or 0.000156% (w/w) PFOS for 28 days. On day 23 of exposure, 100 μg TNP–LPS was injected intravenously into each mouse and when the animals were sacrificed 5 days later, blood samples were collected and serial dilutions of these samples analyzed for their levels of IgM antibodies directed specifically against this antigen employing an ELISA procedure (for further details, see Section 2). The values presented are means ± standard errors ($n = 5$). There were no statistically significant differences between the exposed and control animals, as determined by Mann–Whitney (*U*-test) analysis.

It is important to search for explanations for the differences in the observations made in these three studies. These differences do not seem to be related to the levels of PFOS administered, which were similar and resulted in similar final serum concentrations in all three cases. Indeed, with a TAD of 5 mg/kg the serum levels of PFOS measured in our treated animals (11.6 ± 0.2 μg/ml) were somewhat higher than those reported by Peden-Adams et al. (2008) (expected level = 4.64 μg/ml) and Dong et al. (2009) (measured level = 7.13 μg/ml). If slightly higher serum concentrations were of importance, then we should have obtained more pronounced effects than these other two groups of investigators, which is clearly not the case. Nor do differences in the level of stress caused by exposure help to explain our different findings, since both here and in the study by Dong et al. (2009), no significant change in the serum level of corticosterone, a hormone known to have a negative impact on adaptive immunity (Pruett et al., 1999, 2007), was observed.

The remaining obvious differences between these three studies involve the strain of mice used (B6C3F1 in the case of ourselves and Peden-Adams et al. and C57BL/6 in the investigation by Dong et al.) and route of administration (dietary in our case and by oral gavage in the other two studies). Several studies have revealed that the biological effects including toxicity, bioavailability and metabolism of drugs or toxins administered by oral gavage may differ markedly from the corresponding effects obtained with dietary administration. For instance, Hébert et al. (1994) have demonstrated that dietary administration of cinnamaldehyde allows the delivery of higher doses of this compound without causing the acute toxic effects associated with a gavage bolus administration. Moreover, Yuan et al. (1994) found that the bioavailability of pentachlorophenol is greater upon oral gavage than with dietary administration to rats. In addition, Kapetanovic et al. (2006) have shown that gavage treatment of rats with sulindac results in higher peak and lower trough concentrations of this compound in plasma and mammary tissue, as well as a more pronounced effect on levels of prostaglandin E(2) than are observed with the same dose administered in the diet. Such differences are not surprizing, in light of the

expectation that gavage and dietary administration of a compound would result in different pharmacokinetic patterns.

On the basis of such findings, we propose that the route of administration may have a considerable impact on the immunotoxic effects exerted by PFOS and may explain, at least in part, some of the differences between the observations reported here and those made earlier by Peden-Adams et al. (2008) and Dong et al. (2009). Additional experiments designed to test this proposal are clearly warranted.

In conclusion, the present investigation shows that 28-day exposure of adult male B6C3F1 mice to dietary levels of PFOS that result in serum concentrations one or two orders of magnitude higher than those observed in occupationally exposed workers does not compromise the adaptive immune system. Consistent with this conclusion, Lefebvre et al. (2008) observed that 28-day exposure of adult male Sprague–Dawley rats to a TAD of approximately 37 mg PFOS/kg (serum concentration 13.45 ± 1.48 μg/ml) alters neither the absolute or relative numbers of different thymocyte or splenocyte populations nor the specific humoral IgG response against a foreign antigen (keyhole limpet hemocyanin).

In this connection, although no direct information concerning the effects of PFOS on the human immune system is presently available, medical surveillance of occupationally exposed workers with serum PFOS concentrations ranging between 0.06 and 10.06 μg/ml (ppm) has revealed no substantial changes in hematological parameters, including the white blood cell count, a parameter related to the status of the immune system (Olsen et al., 2003). Moreover, longitudinal analysis of male workers over a period of 6 years revealed no significant alterations in lipid or hepatic clinical chemistry that would be consistent with the known toxic effects of this compound on mice (Olsen et al., 2003). Thus, dietary exposure would appear to be a more realistic model for human exposure than administration by gavage. At the same time, it should be emphasized that humans and other organisms are exposed to PFOS for much longer periods than 28 or even 60 days and, in addition, there may be certain subpopulations of humans and other animals that are particularly susceptible to the immunotoxic effects of this fluorochemical. Obviously, further studies on these issues are required.

Conflicts of interest

None of the authors has any conflicting interests.

Acknowledgments

This study was financed by an unrestricted grant from the 3M Company (St. Paul, Minnesota, USA). We would like to thank Dave J. Ehresman (3M Company) for his skillful determination of the serum levels of PFOS.

References

3M Company, 2003. Environmental and Health Assessment of Perfluorooctanesulfonate and Its Salts. Washington, DC: U.S. Environmental Protection Agency. U.S. EPA docket AR-226-1486.

Bouman, A., Heineman, M.J., Faas, M.M., 2005. Sex hormones and the immune response in humans. Hum. Reprod. Update 11, 411–423.

Butenhoff, J.L., Olsen, G.W., Pfahles-Hutchens, A., 2006. The applicability of biomonitoring data for perfluorooctanesulfonate to the environmental public health continuum. Environ. Health. Perspect. 114, 1776–1782.

Cunningham, A.J., Szenberg, A., 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 14, 599–600.

Curran, I., Hierlihy, S.L., Liston, V., Pantazopoulos, P., Nunnikhoven, A., Tittlemier, S., Barker, M., Trick, K., Bondy, G., 2008. Altered fatty acid homeostasis and related toxicologic sequelae in rats exposed to dietary potassium perfluorooctanesulfonate (PFOS). J. Toxicol. Environ. Health A 71, 1526–1541.

DeWitt, J.C., Copeland, C.B., Luebke, R.W., 2009. Suppression of humoral immunity by perfluorooctanoic acid is independent of elevated serum corticosterone concentration in mice. Toxicol. Sci. 109, 106–112.

Dong, G.H., Zhang, Y.H., Zheng, L., Liu, W., Jin, Y.H., He, Q.C., 2009. Chronic effects of perfluorooctanesulfonate exposure on immunotoxicity in adult male C57BL/6 mice. Arch. Toxicol. 83, 805–815 (Epub 2009 April 3).

Ehresman, D.J., Froehlich, J.W., Olsen, G.W., Chang, S.C., Butenhoff, J.L., 2007. Comparison of human whole blood, plasma, and serum matrices for the determination of perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and other fluorochemicals. Environ. Res. 103, 176–184.

Ericson, I., Martí-Cid, R., Nadal, M., Van Bavel, B., Lindström, G., Domingo, J.L., 2008. Human exposure to perfluorinated chemicals through the diet: intake of perfluorinated compounds in foods from the Catalan (Spain) market. J. Agric. Food Chem. 56, 1787–1794.

Fromme, H., Schlummer, M., Möller, A., Gruber, L., Wolz, G., Ungewiss, J., Böhmer, S., Dekant, W., Mayer, R., Liebl, B., Twardella, D., 2007. Exposure of an adult population to perfluorinated substances using duplicate diet portions and biomonitoring data. Environ. Sci. Technol. 41, 7928–7933.

Fromme, H., Tittlemier, S.A., Völkel, W., Wilhelm, M., Twardella, D., 2009. Perfluorinated compounds – exposure assessment for the general population in Western countries. Int. J. Hyg. Environ. Health 212, 239–270.

Harper, N., Howie, L., Connor, K., Dickerson, R., Safe, S., 1993. Immunosuppressive effects of highly chlorinated biphenyls and diphenyl ethers on T-cell dependent and independent antigens in mice. Toxicology 31, 123–135.

Haughom, B., Spydevold, O., 1992. The mechanism underlying the hypolipemic effect of perfluorooctanoic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and clofibric acid. Biochim. Biophys. Acta 22, 65–72.

Hébert, C.D., Yuan, J., Dieter, M.P., 1994. Comparison of the toxicity of cinnamaldehyde when administered by microencapsulation in feed or by corn oil gavage. Food Chem. Toxicol. 32, 1107–1115.

Heyman, B., Holmquist, G., Borwell, P., Heyman, U., 1984. An enzyme-linked immunosorbent assay for measuring anti-sheep erythrocyte antibodies. J. Immunol. Methods 68, 193–204.

Houde, M., Martin, J.W., Letcher, R.J., Solomon, K.R., Muir, D.C., 2006. Biological monitoring of polyfluoroalkyl substances. Environ. Sci. Technol. 40, 3463–3473.

Hudson, L., Hay, F., 1989. Practical Immunology, 3rd ed. Blackwell Scientific Publication, Oxford, UK.

Ikeda, T., Aiba, K., Fukuda, K., Tanaka, M., 1985. The induction of peroxisome proliferation in rat liver by perfluorinated fatty acids, metabolically inert derivatives of fatty acids. J. Biochem. 98, 475–482.

Kapetanovic, I.M., Krishnaraj, R., Martin-Jimenez, T., Yuan, L., van Breemen, R.B., Lyubimov, A., 2006. Effects of oral dosing paradigms (gavage versus diet) on pharmacokinetics and pharmacodynamics. Chem. Biol. Interact. 164, 68–75.

Keil, D.E., Mehlmann, T., Butterworth, L., Peden-Adams, M.M., 2008. Gestational exposure to perfluorooctane sulfonate suppresses immune function in B6C3F1 mice. Toxicol. Sci. 103, 77–85.

Kissa, E., 1985. Determination of 2H-hexafluoropropanol-2 in air. J. Chromatogr. 319, 407–411.

Kuklenyik, Z., Reich, J.A., Tully, J.S., Needham, L.L., Calafat, A.M., 2004. Automated solid-phase extraction and measurement of perfluorinated organic acids and amides in human serum and milk. Environ. Sci. Technol. 38, 3698–3704.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., Seed, J., 2007. Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol. Sci. 99, 366–394.

Lau, C., Butenhoff, J.L., Rogers, J.M., 2004. The developmental toxicity of perfluoroalkyl acids and their derivatives. Toxicol. Appl. Pharmacol. 198, 231–241.

Lefebvre, D.E., Curran, I., Armstrong, C., Coady, L., Parenteau, M., Liston, V., Barker, M., Aziz, S., Rutherford, K., Bellon-Gagnon, P., Shenton, J., Mehta, R., Bondy, G., 2008. Immunomodulatory effects of dietary potassium perfluorooctane sulfonate (PFOS) exposure in adult Sprague–Dawley rats. J. Toxicol. Environ. Health A 71, 1516–1525.

Lehmler, H.J., 2005. Synthesis of environmentally relevant fluorinated surfactants—a review. Chemosphere 58, 1471–1496.

Luebker, D.J., Case, M.T., York, R.G., Moore, J.A., Hansen, K.J., Butenhoff, J.L., 2005. Two-generation reproduction and cross-foster studies of perfluorooctanesulfonate (PFOS) in rats. Toxicology 215, 126–148.

OECD, 2002. Hazard Assessment of Perfuorooctane Sulfonate (PFOS) and its Salts. ENV/JM/RD(2002)17/Final. Available at: http://www.oecd.org/dataoecd/23/18/2382880.pdf.

Olsen, G.W., Burris, J.M., Burlew, M.M., Mandel, J.H., 2003. Epidemiologic assessment of worker serum perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) concentrations and medical surveillance examinations. J. Occup. Environ. Med. 45, 260–270.

Olsen, G.W., Burris, J.M., Ehresman, D.J., Froehlich, J.W., Seacat, A.M., Butenhoff, J.L., Zobel, L.R., 2007. Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ. Health. Perspect. 115, 1298–1305.

Peden-Adams, M.M., Keller, J.M., Eudaly, J.G., Berger, J., Gilkeson, G.S., Keil, D.E., 2008. Suppression of humoral immunity in mice following exposure to perfluorooctane sulfonate. Toxicol. Sci. 104, 144–154.

Pruett, S., Hebert, P., Lapointe, J.M., Reagan, W., Lawton, M., Kawabata, T.T., 2007. Characterization of the action of drug-induced stress responses on the immune system: evaluation of biomarkers for drug-induced stress in rats. J. Immunotoxicol. 4, 25–38.

Pruett, S.B., Collier, S., Wu, W.J., Fan, R., 1999. Quantitative relationships between the suppression of selected immunological parameters and the area under the corticosterone concentration vs. time curve in B6C3F1 mice subjected to exogenous corticosterone or to restraint stress. Toxicol. Sci. 49, 272–280.

Qazi, M.R., Xia, Z., Bogdanska, J., Chang, S.C., Ehresman, D.J., Butenhoff, J.L., Nelson, B.D., DePierre, J.W., Abedi-Valugerdi, M., 2009a. The atrophy and changes in the cellular compositions of the thymus and spleen observed in mice subjected to short-term exposure to perfluorooctanesulfonate are high-dose phenomena mediated in part by peroxisome proliferator-activated receptor-alpha (PPARalpha). Toxicology 260, 68–76.

Qazi, M.R., Bogdanska, J., Butenhoff, J.L., Nelson, B.D., Depierre, J.W., Abedi-Valugerdi, M., 2009b. High-dose, short-term exposure of mice to perfluorooctanesulfonate (PFOS) or perfluorooctanoate (PFOA) affects the number of circulating neutrophils differently, but enhances the inflammatory responses of macrophages to lipopolysaccharide (LPS) in a similar fashion. Toxicology 262, 207–214.

Seacat, A.M., Thomford, P.J., Hansen, K.J., Clemen, L.A., Eldridge, S.R., Elcombe, C.R., Butenhoff, J.L., 2003. Sub-chronic dietary toxicity of potassium perfluorooctanesulfonate in rats. Toxicology 183, 117–131.

Seacat, A.M., Thomford, P.J., Hansen, K.J., Olsen, G.W., Case, M.T., Butenhoff, J.L., 2002. Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in cynomolgus monkeys. Toxicol. Sci. 68, 249–264.

Tittlemier, S.A., Pepper, K., Seymour, C., Moisey, J., Bronson, R., Cao, X.L., Dabeka, R.W., 2007. Dietary exposure of Canadians to perfluorinated carboxylates and perfluorooctane sulfonate via consumption of meat, fish, fast foods, and food items prepared in their packaging. J. Agric. Food Chem. 55, 3203–3210.

U.S. EPA (U.S. Environmental Protection Agency), 1998. Health Effects Test Guidelines OPPTS 870.7800 Immunotoxicity. Agency. Prevention, Pesticides and Toxic Substances. (7101). EPA 712–C–98-351. Available at: http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-7800.pdf.

U.S. EPA (U.S. Environmental Protection Agency), 2006. SAB Review of EPA's Draft Risk Assessment of Potential Human Health Effects Associated with PFOA and its Salts. Available at: http://yosemite.epa.gov/sab/sabproduct.nsf/WebreportsbyYearBOARD!OpenView.

Verthelyi, D., 2001. Sex hormones as immunomodulators in health and disease. Int. Immunopharmacol. 1, 983–993.

Yuan, J.H., Goehl, T.J., Murrill, E., Moore, R., Clark, J., Hong, H.L., Irwin, R.D., 1994. Toxicokinetics of pentachlorophenol in the F344 rat. Gavage and dosed feed studies. Xenobiotica 24, 553–560.

Zheng, L., Dong, G.H., Jin, Y.H., He, Q.C., 2009. Immunotoxic changes associated with a 7-day oral exposure to perfluorooctanesulfonate (PFOS) in adult male C57BL/6 mice. Arch. Toxicol. 83, 679–689.