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Further evidence for the involvement of inhibition of cell proliferation and development in thymic and splenic atrophy induced by the peroxisome proliferator perfluorooctanoic acid in mice

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Abstract

We recently demonstrated that severe thymic and splenic atrophy occur upon dietary treatment of mice with potent peroxisome proliferators (PPs), e.g. perfluorooctanoic acid (PFOA), WY-14,643, nafenopin, and di(2-ethylhexyl)phthalate (DEHP). In the present study, we investigated this phenomenon further employing a relative inert PP, PFOA. Comparison of the dose-dependencies and time-courses indicated that the peroxisome proliferative effect occurred prior to atrophy of both the thymus and spleen. However, following withdrawal of PFOA from the diet, the weight of the thymus and spleen rapidly returned to normal within 10 and 5 days, respectively, in contrast to the more persistent peroxisome proliferation. Furthermore, the changes in thymus and spleen weight upon PFOA treatment and the following withdrawal from diet paralleled the changes in total thymocyte and splenocyte counts, respectively. It was found previously that the decreases in the thymocyte populations present in the S and G2/M phases, as well as in the number of $CD4^+CD8^+$ cells upon PFOA treatment, were the most dramatic, perhaps reflecting inhibition of thymocyte proliferation in connection with thymocyte development. Here, the recovery of thymocytes began with increases in the populations in these same phases of the cell cycle, with $CD4^+CD8^+$ cells recovering most rapidly, lending further support to our previous hypothesis. The possible relationship of these immunotoxic effects of PPs to the changes they cause in fatty acid metabolism is discussed.

Keywords: Thymic atrophy; Thymocytes; Splenocytes; Peroxisome proliferator; Perfluorooctanoic acid; Immunotoxicology; Mice

1. Introduction

Peroxisome proliferators (PPs) constitute a large family of widespread foreign compounds, including many industrial chemicals (e.g. phthalates and perfluoro fatty acid), agrochemicals (e.g. phenoxyacetic acids), and important clinical drugs (e.g. acetylsalicylic acid and fibrate hypolipidemic agents). The most prominent effects of PPs on rodents include increases in the number and size of peroxisomes, hypertrophy of the liver, and a potent up-regulation of hepatic peroxisomal fatty acid β-oxidation. Prolonged treatment of rodents with PPs results in the formation of liver tumors [1–3].

There is an increasing awareness that certain drugs and other foreign chemicals (e.g. pesticides) can modulate the immune system via direct or indirect mechanisms. Hundreds of journal articles and many books have been devoted to detailed descriptions of the immunotoxicity of drugs and environmental agents [4–7]. Evaluation of possible effects on the immune system is thus an essential aspect of the safety evaluation of new xenobiotics.

In this connection, the thymus and spleen, which both play central roles in the immunological defences of higher animals, are highly sensitive to damage by xenobiotics and are thus routinely examined for immunotoxicological effects [8,9]. However, as a relatively newly recognized class of chemicals, PPs have received little attention in this respect.

Recently, we demonstrated that severe thymic and splenic atrophy occur upon dietary treatment of mice with potent PPs (e.g. perfluorooctanoic acid [PFOA], WY-14,643, nafenopin, and di(2-ethylhexyl)phthalate

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Abbreviations: PFOA, perfluorooctanoic acid; PP, peroxisome proliferator; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; and DEHP, di(2-ethylhexyl)phthalate.

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(DEHP) [10]. In an attempt to elucidate the mechanism(s) underlying this phenomenon, the highly potent and relatively chemically and metabolically inert PP, PFOA [11], was chosen for more detailed study. PFOA is a representative of the perfluorinated carboxylic acids used as commercial wetting agents and flame-retardants. In earlier studies, we extensively characterized PFOA as a potent and persistent PP [12].

In the present study, we examine whether the immunotoxic effects of PPs are related to or independent of their peroxisome proliferative effects. In this connection, we have characterized the dose- and time-dependencies of peroxisome proliferation and atrophy of the thymus and spleen in mice treated with PFOA, as well as the recovery of the thymus and spleen after withdrawal of this compound from the diet. The cellularity, cellular phenotypes, and cell cycle in the thymus and spleen were also analyzed, revealing that the immunotoxic effect is due, at least in part, to inhibition of thymocyte proliferation.

2. Materials and methods

2.1. Chemicals

PFOA (Aldrich Chemical Co.) and Earle's balanced salt solution (BSS) (Life Technologies) were purchased from the sources indicated. The complete PBS solution (pH = 7.4) consisted of PBS containing 2% FBS and 0.01% NaN_3. All other materials and chemicals were obtained from common commercial sources.

2.2. Animals and treatment

Male C57B1/6 mice (B&K Universal AB) weighing 24–28 g (about 8–10 weeks old) were housed and treated in steel cages in groups of four. These animals were maintained on a 12-hr light/dark cycle at 25° and given free access to laboratory chow R3 containing 5% fat, 24% protein, and 49% carbohydrate (Astra Ewos AB). The PFOA-containing diet was prepared as described previously [12]. Briefly, PFOA was dissolved in 20 mL acetone and mixed with 500 g powdered food to prepare a diet containing 0.001%–0.05% PFOA (w/w). This chow was dried in a ventilated hood until no smell of acetone was detectable (>24 hr). Because mice scatter powdered food, both the normal and PFOA-containing chows were prepared in the form of pellets to allow determination of food intake. The mice were also weighed each day.

For investigation of the dose dependency of the effects of PFOA on these lymphoid organs, mice received diets containing 0.001–0.05% PFOA (w/w) for 10 days. For examination of the time-course, a diet containing 0.02% PFOA was administered for 2–10 days. To monitor recovery, the animals were fed a diet containing 0.02% PFOA (w/w) for 7 days, after which they were given normal diet for 2–10 days.

At the end of the feeding period, the mice were killed by ether inhalation to avoid coagulation of the blood. The liver, thymus, and spleen were dissected out and weighed. The liver samples were then homogenized and subfractionated as described earlier [13].

2.3. Assay of peroxisomal acyl-CoA oxidase

Acyl-CoA oxidase [14] was assayed fluorimetrically with lauroyl-CoA or pamitoyl-CoA as substrate using the hepatic mitochondrial subfraction from both control and PFOA-treated mice.

2.4. Preparation of cell suspensions

Thymocytes and splenocytes were isolated from normal and treated mice by teasing the relevant organ gently with a forceps in BSS. Erythrocytes present in the splenocyte preparations were lysed with 0.83% NH_4Cl–Tris buffer (pH 7.6). All cell suspensions were washed three times and re-suspended in cold BSS prior to analysis.

2.5. Cellularity and cell viability

The cells were counted using a haemocytometer. Cell viability (evaluated on the basis of trypan blue exclusion) was always >95%.

2.6. Staining of cellular DNA with propidium iodide

Single-cell suspensions of thymocytes or splenocytes were stained with propidium iodide (PI) according to a reported procedure [15]. Briefly, 10^6 thymocytes were washed twice with complete PBS and then fixed in 2 mL 70% ice-cold ethanol at 4° for 60 min. Subsequently, one more wash with 1 mL complete PBS and resuspension in 0.5 mL of this same solution were performed. RNase (0.5 mL) (1 mg/mL in PBS) and 1 mL PI (100 μg/mL) were then added and the cells incubated for 20 min at room temperature in the dark. Thereafter, the cells were stored at 4° in the dark until analysis.

2.7. Immunofluorescent staining

Single-cell suspensions of thymocytes or splenocytes (2.0×10^6) suspended in 100 μL cold (4°) complete PBS were incubated in the dark on ice for 20 min with FITC- or Cy-chrome-conjugated monoclonal anti-mouse antibodies (MoAbs) towards CD3, CD4, CD8a, or CD19 (PharMingen). In the case of dual-parameter analysis, two of these MoAbs were present in same incubation. After two washes with cold complete PBS, all samples were fixed in 2% paraformaldehyde (in PBS) for 20 min on ice. After two

additional washes in cold complete PBS, the stained cells were analyzed by flow cytometry.

2.8. Flow cytometric analysis

Cells were analyzed using a single laser FACSCalibur cytometer (Becton Dickinson) with excitation at 488 nm. The data were collected and analyzed employing CellQuest™ Software. For analysis of cell phenotype markers, the lymphocyte populations were gated on the basis of the forward scatter (FSC) and the side-scatter (SSC) signals collected in the linear mode, i.e. aggregates of cells were gated out. The two fluorescent signals were analyzed on a logarithmic scale and data are presented as the percentage of the total cell population exhibiting the fluorescent signal of interest. For cell cycle analysis, the pulse area versus pulse width approach was applied. In this case, doublets and larger aggregates had to be gated out from the single cell population. The data are presented as histograms of cell number versus DNA content. For each sample, 10,000 cells were analyzed.

2.9. Statistical analysis

Each experimental group contained 4–8 animals. Data are presented as means ± SD and the results of statistical analysis using the Student's *t*-test are given where appropriate.

3. Results

3.1. General observations and dose- and time-dependencies of the hepatic hypertrophy, peroxisome proliferation, and atrophy of thymus and spleen induced by PFOA

Dietary administration of PFOA to mice for 10 days resulted in no apparent signs of toxicity, such as sores, poor grooming, lethargy, or other behavioral changes. However, at higher doses, a significant decrease in body weight was observed, which is a well-known effect of potent PPs [12, 16,17]. It has been reported that DEHP reduces body weight significantly in wild-type mice, but has no such effect on PPAR alpha-null mice [17]. Experiments presently ongoing in our laboratory indicate that this decrease in body weight is due to specific loss of adipose tissue, which reflects the influence of PPs on lipid metabolism.[1]

In addition, no significant decrease in total food intake during PFOA treatment was observed, which is in agreement with earlier observations in our laboratory [12]. However, it is worth noting that a significant decrease (−22%) in total water intake was observed in this same experiment, due primarily to the fact that the animals drank less on the first and second days of treatment (data not shown). In this context, the up-regulation of fatty acid β-oxidation caused by PFOA may lead to more endogenous water production in the body, thus decreasing the requirement for intake.

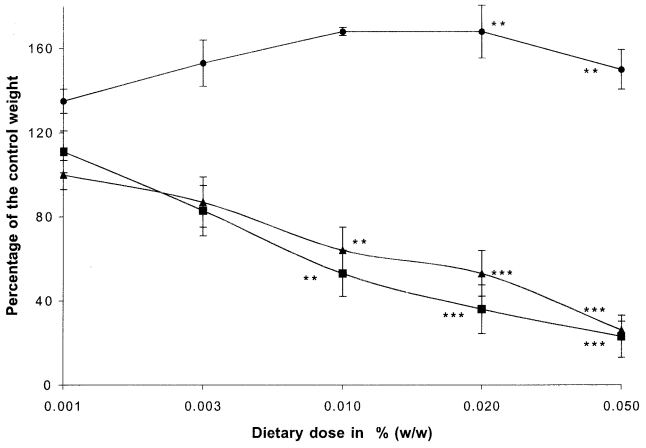

Fig. 1. Dose-dependency of the changes in liver, thymus, and spleen weights during PFOA treatment for 10 days. (●): Liver; (▲): spleen; (■): thymus. The weights are expressed as percentages of the control values and the dosage on a logarithmic scale. The results shown are means ± SD for four animals. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to the control group.

As expected, when mice received dietary PFOA at the indicated doses (Fig. 1) for 10 days, significant increases in liver weight and peroxisome proliferation were observed in all cases. The increase in liver weight and peroxisome proliferation (measured as induction of acyl-CoA oxidase with lauroyl-CoA or palmitol-CoA as substrate) began at the lowest dose and reached their maximal values at a dose of 0.003–0.01% (Figs. 1 and 2). In contrast, the decreases in

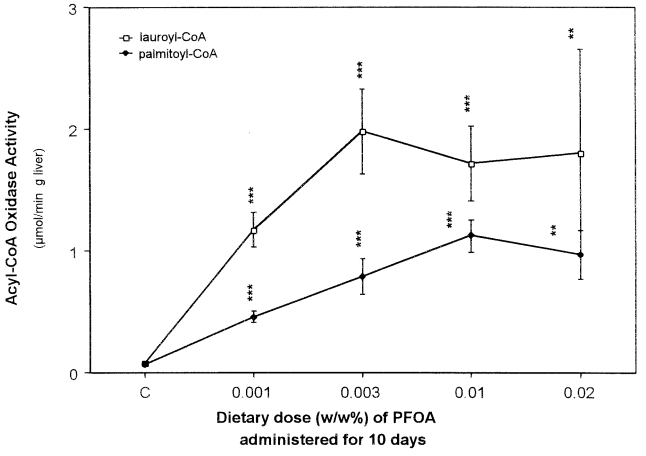

Fig. 2. Response of peroxisomal acyl-CoA oxidase to 10 days of treatment with different doses (w/w) of PFOA. Acyl-CoA oxidase activity with palmitoyl-CoA (●) or lauroyl-CoA (□) as substrate was assayed in the mitochondrial subfraction after 10 days exposure to 0–0.02% (w/w) PFOA. $**P < 0.01$, $***P < 0.001$ compared to the control value.

[1] Xie Y, Yang Q, Nelson BD, DePierre JW, manuscript submitted for publication.

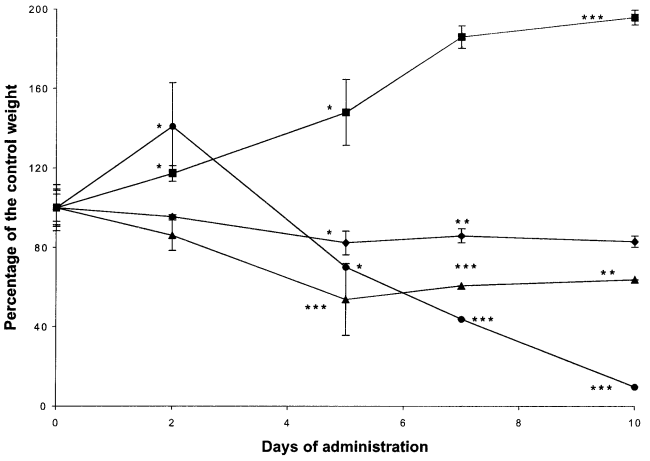

Fig. 3. Time-course of the changes in liver, thymus, and spleen weights during treatment with 0.02% (w/w) PFOA for as long as 10 days. (◆): Body; (■): liver; (▲): spleen; (●): thymus. The weights are expressed as percentages of the control values. The results shown are means ± SD for four animals. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to the control group.

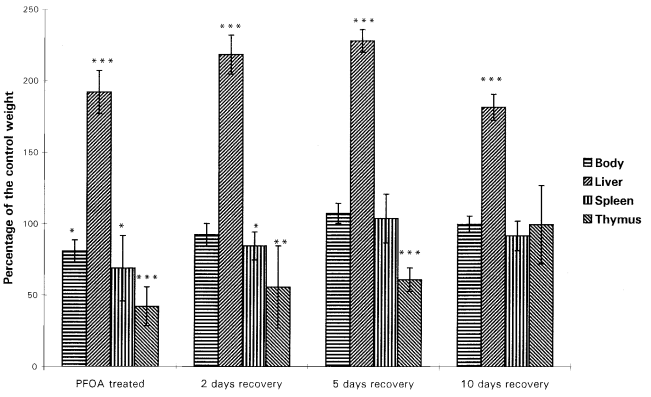

Fig. 5. Changes in body, liver, thymus, and spleen weights upon treatment of mice with 0.02% (w/w) PFOA for 7 days and subsequent withdrawal of this compound from the diet for another 10 days. The weights are expressed as percentages of the control values. The results shown are means ± SD for four animals. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the control group.

the weights of the thymus and spleen began at a higher dose (0.01%) and no maximum was reached in the dose range employed here (Fig. 1).

Upon administration of 0.02% (w/w) PFOA for 1, 2, 5, 7, or 10 days, a significant increase in liver weight was observed even at the earliest time point, with the maximal value being reached after 7 days (Fig. 3). Similarly, hepatic acyl-CoA oxidase was significantly induced even on the first day, and thereafter increased continuously up to 10 days of treatment (Fig. 4). In contrast, significant decreases in thymus and spleen weight were observed only after 5 days of treatment, following which the spleen weight remained constant, while the thymus weight continued to decrease (Fig. 3). Upon prolonged treatment for one month, no further significant decreases in thymus and spleen weight were observed (data not shown). As seen here and earlier, the atrophy of the thymus in response to PFOA treatment is more severe than the atrophy of the spleen. In order to cause minimal effects on body weight, while still having significant effects on the spleen and thymus, treatment with 0.01% PFOA for 10 days (Fig. 1) or 0.02% PFOA for 5–7 days (Fig. 3) was subsequently used routinely.

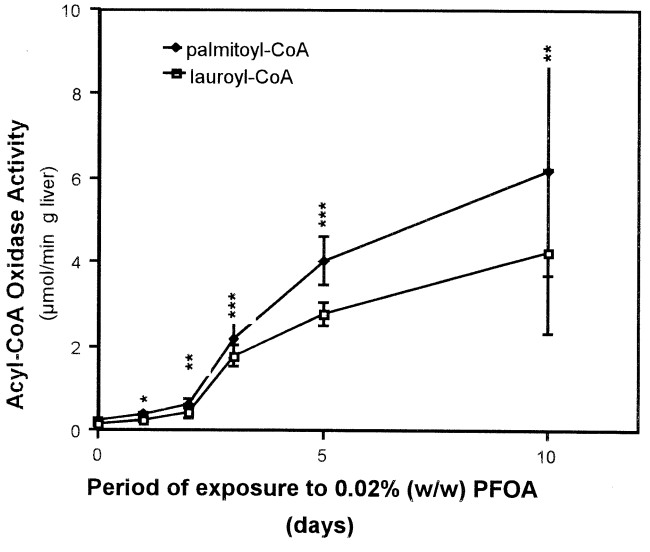

Fig. 4. Acyl-CoA oxidase activity after different periods of exposure to 0.02% (w/w) PFOA. Acyl-CoA oxidase activity towards palmitoyl-CoA (●) or lauroyl-CoA (□) as substrate was assayed in the mitochondrial subfraction after 0, 1, 2, 3, 5, and 10 days of exposure to 0.02% PFOA. $*P < 0.1$, $**P < 0.01$, $***P < 0.001$ compared to the control value.

3.2. Effect of PFOA administration and subsequent withdrawal on body, liver, thymus, and spleen weights

After dietary exposure to 0.02% PFOA for 7 days, the animals received normal chow for an additional 10 days. As seen in Fig. 5, rapid recovery of the body weight was observed from the second day after withdrawal of PFOA from the diet. However, the liver weight did not return to normal even after 10 days of recovery. In our earlier study, even after 20 days of recovery, the activity of lauroyl-CoA oxidase was still more than 6-fold higher than in the control animals [12]. Thymus recovery started at day 2 and was complete after 10 days. The spleen weight returned to normal within 2 days (Fig. 5). Thus, in contrast to the persistent peroxisome proliferation [12], the atrophy of the thymus and spleen caused by PFOA was rapidly reversible.

3.3. Effects of PFOA administration and its subsequent withdrawal on the cellularity and cell surface phenotype of thymocytes and splenocytes

After administration of 0.02% PFOA to mice for 7 days, thymocytes and splenocytes were isolated and significant decreases (by approximately 90 and 70%, respectively) in

Table 1A
Effects of PFOA administration and subsequent withdrawal on the cellularity of antigenically distinct subpopulations of thymocytes in mice

Treatment	Total cell number		CD4+CD8+		CD4+		CD8+		CD4−CD8−	
	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)
None (control)	56.8 ± 13.5	(100)	40.8 ± 12.9	(100)	2.27 ± 0.49	(100)	3.27 ± 0.82	(100)	7.49 ± 1.22	(100)
PFOA[a]	5.81 ± 3.86***	(10)	1.73 ± 1.89***	(4)	0.87 ± 0.59*	(38)	0.75 ± 0.46**	(23)	2.46 ± 1.43**	(33)
2 days recovery	3.23 ± 0.97***	(6)	0.11 ± 0.10***	(0.3)	0.16 ± 0.10***	(7)	0.19 ± 0.16***	(6)	2.76 ± 0.74***	(37)
5 days recovery	11.9 ± 4.91**	(21)	7.23 ± 3.67**	(18)	0.31 ± 0.11***	(14)	0.57 ± 0.24***	(17)	3.84 ± 1.61*	(51)
10 days recovery	59.5 ± 20.8	(105)	45.8 ± 16.1	(112)	2.23 ± 0.96	(98)	2.93 ± 1.34	(90)	8.55 ± 3.53	(114)

[a] 0.02% (w/w), 7 days.

All values are means ±SD for four animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group. Other conditions were as described in the Materials and methods.

the total numbers of these cells noted (Table 1, A and B). Following withdrawal of PFOA, neither thymocytes nor splenocytes showed any change in number during the first 2 days, but these cell numbers returned rapidly to normal during days 5–10 of recovery (Table 1, A and B). Comparison of the changes in cell numbers and tissue weights of the thymus and spleen induced by PFOA and during recovery (Table 1, A and B and Fig. 5) reveals that the decrease in cell number was larger than the loss of tissue weight. In contrast, tissue weight recovered more rapidly than did cell number, as can be clearly seen after 2 days of recovery.

In order to determine the effect of PFOA on phenotypically distinct cell subpopulations, we examined CD4 and CD8 expression by thymocytes and CD3 and CD19 expression by splenocytes, employing two-color flow cytometry. The results (Table 1, A and B) reveal that PFOA affected phenotypically distinct thymocytes to different extents: the number of $CD4^+CD8^+$ cells decreased by 95%; $CD4^-CD8^-$ cells decreased by 65%; and $CD4^+$ and $CD8^+$ cells decreased by 65% and 75%, respectively. Following withdrawal of PFOA, all of these populations were seen to be on the way to recovery by day 5 and had completely recovered after 10 days. However, during the first 2 days of recovery, the numbers of $CD4^+CD8^+$, $CD4^+$, and $CD8^+$ cells actually decreased even further.

In the case of splenocytes (Table 1B), decreases in the numbers of both T-cells ($CD3^+$; −65%) and B-cells ($CD19^+$; −75%) occurred upon PFOA treatment. Among the T-cells, significant decreases in both T-helper ($CD4^+$, −65%) and T-cytotoxic ($CD8^+$, −65%) cells were observed (Table 1B). Following withdrawal of PFOA, both T- and B-cells and T-helper and T-cytotoxic cells recovered in number, this process beginning at day 5 and being complete after 10 days.

3.4. Effects of PFOA administration and subsequent withdrawal on the cell cycle of thymocytes and splenocytes

In order to determine whether the decreased numbers of thymocytes and splenocytes observed after PFOA treatment reflect a decrease in the percentage of the cells carrying out DNA synthesis (e.g. cell proliferation), we examined the cell cycle in thymocytes and splenocytes using a flow cytometric technique based on binding of the dye propidium iodide to DNA. This approach allowed us to measure the percentage of the cells that were in the G0/G1, S, and G2/M stages of the cell cycle. Upon administration of PFOA to mice for 7 days, significant decreases (35% and 65%, respectively) in the proportions of thymocytes in the S and G2/M phases and significant increases in the G0/G1 phases were observed (Table 2). Upon subsequent withdrawal of PFOA from the diet, the proportion of the cells in the S-phase and G2/M phases returned to normal within 2–5 days, whereas the G0/G1 phase was normalized within 5–10 days. This finding indicates that the decrease in the number

Table 1B
Effects of PFOA administration and subsequent withdrawal on the cellularity of antigenically distinct subpopulations of splenocytes in mice

Treatment	Total cell number		CD3+		CD4+		CD8+		CD19+	
	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)
None (control)	33.8 ± 11.8	(100)	9.74 ± 3.03	(100)	7.21 ± 1.63	(100)	4.56 ± 1.66	(100)	12.3 ± 3.85	(100)
PFOA[a]	9.18 ± 2.11**	(27)	3.23 ± 0.73**	(33)	2.32 ± 0.88**	(32)	1.70 ± 0.44*	(37)	3.13 ± 0.59**	(25)
2 days recovery	10.6 ± 2.76*	(31)	3.64 ± 0.82**	(37)	2.36 ± 0.56***	(33)	1.80 ± 0.48*	(39)	3.13 ± 2.41**	(25)
5 days recovery	14.6 ± 3.42*	(43)	4.28 ± 1.07*	(44)	3.12 ± 0.92**	(43)	2.15 ± 0.49*	(47)	5.53 ± 1.11*	(45)
10 days recovery	25.2 ± 3.80	(75)	7.86 ± 1.77	(81)	5.96 ± 1.40	(83)	3.99 ± 0.56	(88)	9.05 ± 1.00	(74)

[a] 0.02% (w/w), 7 days.

All values are means ±SD for four animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group. Other conditions were as described in the Materials and methods.

Table 2
Effects of PFOA administration and subsequent withdrawal on the cell cycle of thymocytes in mice

Treatment	G1/2 phase	(%)	G0/G1 phase	(%)	S phase	(%)	G2/M phase	(%)
None (control)	6.41 ± 0.67	(100)	69.4 ± 1.07	(100)	4.01 ± 0.31	(100)	19.5 ± 0.58	(100)
PFOA[a]	10.5 ± 2.15*	(164)	80.1 ± 4.50**	(115)	2.58 ± 0.57**	(64)	6.64 ± 2.76***	(34)
2 days recovery	6.74 ± 2.99	(105)	84.9 ± 2.79***	(122)	3.93 ± 0.38	(98)	4.32 ± 0.15***	(22)
5 days recovery	2.25 ± 0.56***	(35)	75.7 ± 4.61*	(109)	4.40 ± 0.22	(110)	16.8 ± 4.40	(86)
10 days recovery	2.64 ± 0.33***	(41)	72.1 ± 4.02	(104)	4.18 ± 0.29	(104)	20.3 ± 3.86	(104)

[a] 0.02% (w/w), 7 days.

All values (given as percentages of total fluorescence) are means ± SD for four animals. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the control group. Other conditions were as described in the Materials and methods.

of thymocytes caused by PFOA treatment is due mainly, if not entirely, to inhibition of thymocyte proliferation. In contrast, no changes in the cell cycle were observed in splenocytes after PFOA treatment, as previously described [10].

4. Discussion

Accumulating evidence indicates that the immune system can be a major target for toxic effects of xenobiotics [4–7,19]. We recently reported that severe thymic and splenic atrophy occur upon dietary treatment of mice with potent PPs (e.g. perfluorooctanoic acid (PFOA), WY-14,643, nafenopin, and di(2-ethylhexyl)phthalate (DEHP) [10].

In attempt to characterize the signals which initiate these changes, the relative dose-dependencies and time-courses for the changes in liver, thymus, and spleen weights, as well as for peroxisome proliferation upon dietary treatment of mice with PFOA, were of interest. Our results demonstrate that both initiation as well as achievement of the maximal effects of liver hypertrophy and peroxisome proliferation require lower doses than do the decrease in thymus and spleen weights. Liver hypertrophy and peroxisome proliferation also begin more rapidly after commencement of treatment than do the decreases in thymus and spleen weights. These findings indicated that peroxisome proliferation occurs prior to the immunotoxic effect on these lymphoid tissues. However, in contrast to the persistence of the increase in liver weight and peroxisome proliferation after withdrawal of PFOA, rapid recovery of normal thymus and spleen weights is observed.

Chemical-induced immunotoxicity can be organ and/or cell-specific [19]. Phenotypic and cell cycle analysis of thymocytes demonstrated that, although all cell populations are decreased dramatically in number by PFOA, the largest effect is on the immature $CD4^+CD8^+$ population, which are also the cells that begin to recover first. In the earliest stages of their development, thymocytes express neither CD4 nor CD8, after which these cells differentiate to become $CD4^+$ $CD8^+$ thymocytes. These double-positive cells then undergo a rigorous process of proliferation and selection. Cells that survive this process down-regulate either their CD4 or CD8 molecules to become single-positive ($CD4^+$ or $CD8^+$) cells, which are then released into the periphery [7,9,18]. Thus, the more pronounced effect on $CD4^+$ $CD8^+$ cells indicates that PFOA may interfere with the process of thymocyte proliferation. The significant decreases in the proportions of thymocytes present in the S and G2/M phases upon PFOA treatment, as well as the fact that recovery of the thymocytes begins with increases in the cells in the S and G2/M phases, support this hypothesis.

Phenotypic analysis of splenocytes revealed that the total numbers of B- and T-cells decrease in parallel in connection with PFOA treatment. Furthermore, the recovery of T- and B-cells also occurs in parallel. These findings might indicate that B-cell maturation and/or differentiation in the bone marrow is also influenced by PFOA treatment. In comparison with the changes in thymocyte number, the smaller change in splenocyte number might be explained by the fact that T- and B-cells in the spleen do not proliferate without stimulation by antigens. The splenic atrophy may simply reflect a decrease in the supply of T- and B-cells provided by the thymus and bone marrow.

The decreases in thymocyte and splenocyte numbers observed upon PFOA treatment were more pronounced than the corresponding decreases in organ weight. This may indicate that the matrix structures of the spleen and thymus are little affected, a question which we are now examining histologically and immunohistochemically. Furthermore, the rapid recovery of thymus and spleen weights following withdrawal of PFOA indicates little effect on the stem cells, a question which will be addressed further by examining bone marrow stem cells.

It is always necessary to ask whether alterations in lymphoid organ or cells in response to exposure to xenobiotics lead to changes in immunological functions [4,6,9,19]. We have recently demonstrated that PFOA treatment causes severe suppression of the specific humoral immunological response to horse red blood cells in mice and that *ex vivo* splenocyte proliferation in response to T- and B-cell activators is also reduced by *in vivo* treatment of mice with PFOA.[2]

[2] Yang Q, Abedi-Valugerdi M., Xie Y., Zhao X-Y., Möller G., Nelson B. D., DePierre J. W., manuscript submitted for publication.

We have previously shown that PFOA (1–200 μM, similar to the plasma level reached in mice receiving diet containing 0.02% PFOA) does not exert direct effects on thymocytes and splenocytes *in vitro* [10]. Moreover, secondary effects on the immune system as a consequence of primary effects on other organs are well known [4,9,19]. It is well established that glucocorticoid, produced by the adrenal glands, inhibits and/or kills immature thymocytes *in vivo* [20]. In addition, it has been demonstrated that thymic atrophy induced by a number of chemicals is associated with increased levels of glucocorticoids [21–23]. However, previous investigations have shown that other PPs (e.g. nafenopin and clofibrate) do not increase the concentration of corticosterone in the blood of rodents [24,25]. Thus, it appears unlikely that the thymic and splenic atrophy caused by PFOA and other PPs in mice is due to effects on circulating levels of cortisone.

Among other factors, the immune system must be generally supplied with fatty acids for its development and physiological responses, because the cells of immune tissues do not synthesize fatty acids themselves [26–28,30, 32]. The physiological significance of lipids, and especially of plasma lipoproteins and cholesterol, for the immune system has been described [26–37]. Even in the case of human immune responses, significant differences between hypo- and hypercholesterolemia have been observed [33].

It is now well established that PPs exert their pleiotropic effects on fatty acid metabolism via so-called peroxisome proliferator—activated receptors (PPARs) [1,3,38–43]. These effects include hepatomegaly, an increase in hepatic uptake and β-oxidation of fatty acids, as well as alteration of the expression of fatty acid transport proteins (e.g. fatty acid binding protein and lipoprotein) and acyl-CoA synthase [44–47]. Since PPs cause peroxisome proliferation prior to their effects on the thymus and spleen, our working hypothesis is that these compounds exert their effects on the immune system via altering fatty acid transport and/or metabolism. In this context, experiments presently ongoing in our laboratory have demonstrated that PP treatment reduces the circulating level of lipids, which might result in inhibition of thymocyte and lymphocyte proliferation.

The molecular mechanism(s) underlying the ability of PPs to cause atrophy of the thymus and spleen in rodents is now under further investigation in our laboratory. Such additional studies may help elucidate a new mechanism by which non-genotoxic compounds such as PPs cause tumors, as well as revealing that PPs increase susceptibility to infection by bacteria, viruses, fungi, and parasites. This information will improve our ability to assess the possible risks posed by PPs to human health.

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