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THE OHIO STATE UNIVERSITY

Investigation of the Hepatotoxic and Immunotoxic Effects of the Peroxisome Proliferator Perfluorodecanoic Acid

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Air Force Office of Scientific Research
Bolling Air Force Base, D.C. 20332

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These studies examine the immunotoxic effects 8 days and 30 days following an intraperitoneal injection of PFDA. Histologic alterations observed in lymphoid organs at 8 days following PFDA treatment were more severe than those apparent at 30 days following PFDA treatment. Relative thymus weight was decreased at both 8 and 30 days following PFDA treatment, whereas relative spleen weight was decreased only at 8 days following PFDA treatment. Proliferation in response to Con A in preliminary 30 day and in vitro PFDA studies was decreased. IgG_{2a} production, but not IgA or IgM production was decreased at 8 but not 30 days following PFDA treatment. DTH response at 8 and 30 days after PFDA treatment tended to be suppressed, although these data were not statistically significantly different. NK activity was increased at 30 but not 8 days following PFDA treatment and appeared to be anorexia-associated. Preliminary data on IL-2R, and spleen and thymic cell populations have been collected. Initial experiments have begun on the effect of PFDA on induction of hepatic Enoyl CoA hydratase.

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I. STATEMENT OF WORK

The following is a list of specific aims for which work has been completed or has been ongoing following the April 1991 Annual Technical Report.

A. Evaluation of Immunotoxic Effects of PFDA 8 days and 30 days following PFDA treatment.

1. Morphologic changes of lymphoid organs 8 days and 30 days following PFDA treatment have been completed.

2. The effect of PFDA on lymphocyte proliferation has been completed for 8 days and partially completed for 30 days following PFDA treatment.

3. The effect of PFDA on delayed type hypersensitivity has been completed for 8 days and 30 days following PFDA treatment.

4. The effect of PFDA on natural killer cell function has been completed for 8 days and 30 days following PFDA treatment.

5. The effect of PFDA on antigen specific antibody production has been completed for 8 days and 30 days following PFDA treatment.

6. Samples have been collected for the measurement of IL-2 production for 8 days and 30 days following PFDA treatment.

7. The majority of experiments evaluating percentages of B cells and $CD4^+$ versus $CD8^+$ T cells in the spleens of PFDA treated rats have been completed for 8 days and 30 days following PFDA treatment.

8. Approximately half of the experiments on $CD4^+$, $CD8^+$, and double positive cells in the thymuses of rats treated with PFDA for 8 days or 30 days have been completed.

9. Two experiments on IL-2 receptor expression in isolated spleen cells 8 days or 30 days following PFDA treatment have been completed.

10. The evaluation of the effect of PFDA on IL-1 and PGE-2 production is in progress.

11. The evaluation of the effect of PFDA on MHCII expression, capping, phagocytosis, and respiratory burst is in progress.

B. Immunotoxic effects of in vitro PFDA treatment.

1. A subtoxic PFDA concentration range has been established.

2. The majority of experiments on the effect of PFDA on natural killer activity have been completed.

3. The evaluation of the effect of PFDA on proliferation in response to mitogens is in progress.

4. The evaluation of the effect of PFDA on IL-1, IL-2, and PGE-2 production is in progress.

5. The evaluation of the effect of PFDA on MHCII expression, capping, phagocytosis, and respiratory burst is in progress.

C. Hepatotoxic effects of PFDA treatment.

1. Characterization of the kinetics of peroxisomal proliferation in PFDA treated hepatocytes is in progress.

2. Production of polyclonal antibodies to the PFDA-induced enoyl coA hydratase is in progress.

3. Characterization of the kinetics of induction of cytochrome P-450 and enoyl coA hydratase upon PFDA treatment is in progress.

4. The effects of PFDA on hepatocyte surface IgA receptor expression will be determined.

II. RESEARCH STATUS

This report summarizes work which has been completed April 1991 - April 1992, and describes work in progress.

A. Immunotoxic Effects of PFDA

1. Morphologic changes and organ weight changes 30 days following PFDA treatment.

a. Rationale

Thymic atrophy and hepatocellular hypertrophy were observed in 8-day PFDA studies as reported in the previous year. Morphologic evaluation at 30 days following PFDA treatment demonstrates which organs sustain long-term toxic effects.

b. Methods

Lymphoid organs, kidney and liver were removed asceptically, and weighed. A small section was removed and fixed in formalin. Fixed tissues were dehydrated, embedded in paraffin, sectioned at 5-6 μm and stained with hematoxylin and eosin.

c. Results

1. Histopathology

Unlike the 8-day studies, rats in the 30-day study failed to show any significant histologic alterations in any of the lymphoid organs (thymus, spleen, mesenteric lymph node, of Peyers Patch). Although grossly the thymus was markedly reduced in size in the 50 mg/kg treated rats, the cellularity and cortico-medullary ratio appeared normal. This may reflect recovery of the thymus following earlier atrophy or depletion. The liver showed similar changes to those of rats in the 8-day study (hepatocellular hypertrophy and increased granularity in the 50 mg/kg group). However, the changes were not as striking or consistent as in the rats treated for 8 days. Mild to moderate hepatocellular atrophy was noted in the pair-fed control rats for both the 20 mg/kg and 50 mg/kg treated rats.

2. Organ Weights

Relative liver weight was increased in PFDA-treated rats by 50% (20 mg/kg) and by 100% (50 mg/kg) of *ad libitum* control values (Figure 1). Relative kidney weight was increased by 50% in 50 mg/kg PFDA-treated rats when compared to *ad libitum* control values, however 20 mg/kg PFDA-treated rats did not have altered relative kidney weights (Figure 2). Relative thymus weight was significantly decreased by 21% in 50 mg/kg PFDA-treated rats when compared to *ad libitum* control values (Figure 3). Relative spleen weight was not significantly altered when compared to *ad libitum* control values (Figure 4).

d. Significance

Changes in lymphoid organs were most marked 8 days following PFDA treatment. Although relative thymus weight was still decreased at 30 days following PFDA treatment, relative spleen weight was not different from control values and morphological changes were not apparent. These data indicate that recovery is occurring in the lymphoid organs following the initial insult and that damage to the thymus is of longer-term duration than

damage to the spleen.

2. Proliferation of lymphocytes in response to mitogenic stimulation following PFDA treatment 30 days in vivo, or 72 hours in vitro.

a. Rationale

This assay is used to determine if lymphocytes are capable of proliferating normally in response to T cell mitogen, B cell mitogen or antigen following PFDA treatment.

b. Methods

1. 30 day experiments

Fischer 344 rats were treated with a single intraperitoneal dose of PFDA 30 days prior to sacrifice, and were immunized with KLH 14 and 7 days prior to sacrifice. Spleens were isolated and single cell suspensions were prepared. Mononuclear cells were isolated by Ficoll density centrifugation. Cultures containing 2×10^5 cells/well were stimulated with Con A (T cell mitogen), LPS (B cell mitogen), or KLH (antigen to which the rats were immunized). Cultures were incubated for 72 hours, and ^{3}H-thymidine incorporation was determined during the last 6 hours of cell culture.

2. In vitro experiments

Spleen cells were isolated from untreated Fischer 344 rats and were cultured as described above in the presence of 0 - 150 μM PFDA in 0.1% propylene glycol, or 0.1% propylene glycol alone.

c. Results

1. 30 day experiments

Preliminary evidence indicates a slight decrease in Con A induced lymphoproliferation upon PFDA treatment (Figure 5). An increase in LPS-induced and KLH-induced proliferation is observed upon PFDA treatment (data not shown). Final experiments and statistical analyses of data will be performed in the next 2 months.

2. In vitro experiments

In three out of four experiments, suppression of the Con A-induced T cell proliferation was observed. Table I shows a representative experiment where suppression of up to 68% was observed without a significant change in viability. Viability of cultures containing up to 100 μM PFDA is not significantly altered, but viability decreases dramatically at higher concentrations.

d. Significance

Preliminary data indicate that T cell proliferation in response to Con A is decreased following in vivo (30 day) and in vitro treatment with PFDA when compared to controls. B cell proliferation and antigen-induced proliferation are apparently increased 30 days following in vivo PFDA treatment. These data indicate that T cell activation may be impaired 30 days following PFDA treatment, whereas B cell activation is increased. More experimentation is needed to be conclusive.

3. Effect of PFDA on DTH responsiveness 8 and 30 days following PFDA treatment.

a. Rationale

This experiment addresses the effect of PFDA on cellular immune responses involving T cell induced macrophage recruitment and activation. Cellular immune responses are improtant in defending the host against viral and parasitic infections.

b. Methods

Fischer 344 rats received a single injection of PFDA 8 days or 30 days prior to sacrifice, and were immunized with KLH 14 and 7 days prior to sacrifice. These rats were injected with KLH in one footpad and saline in the other footpad 24 hours prior to sacrifice. Footpad swelling, the difference in millimeters between KLH-treated and saline-treated footpads was determined.

c. Results

PFDA causes a trend of decreased delayed type hypersensitivity 8 days and 30 days following PFDA treatment, which is not statistically significant (Figure 6).

d. Significance

Cellular immunity appears to be impaired following PFDA treatment. The mechanism of this impairment is not known at this time. T cell activation has been shown to be decreased following PFDA treatment in preliminary experiments, but an effect of PFDA on macrophage recruitment and activation cannot be ruled out. Due to the insensitivity of this assay, future experiments utilizing the mixed lymphocyte response are recommended.

4. Effect of PFDA on NK cell Activity

a. Rationale

NK activity is important in innate immunity against viral infections and in tumor surveillance, therefore the effect of PFDA on NK activity is being determined.

b. Methods

Rats were treated and spleen cells (effector cells) were isolated as described previously. Macrophages were removed by an adherence step. Target cells (YAC-1 mouse lymphoma cells) were stained with PKH-2, a viable fluorescent membrane dye. Cultures containing 100:1, 50:1 or 25:1 effector cells:target cells were prepared. Following a conjugation step, cultures were incubated at 37°C for 4 hours. Cells were pelleted and half of the supernatant was removed. This was replaced by an equal volume of25 μg/ml propidium iodide. % cytotoxicity was determined by incorporation of propidium iodide into dead target cells as assessed by flow cytometry.

c. Results

1. <u>8 day experiments</u>.

No significant changes in NK activity were observed 8 days following PFDA treatment (Table II).

2. <u>30 day experiments</u>.

NK activity was significantly increased 30 days following PFDA treatment by 19% at the 100:1 E:T ratio (50 mg/kg) and by 16% at the 25:1 ratio (20 mg/kg and 50 mg/kg) (Table III). Pair-fed NK activity was also significantly greater than *ad libitum*-fed control values at all E:T ratios.

3. In vitro experiments.

The majority of experiments have been completed. A slight increase in NK activity has been observed at 60 μM and 40 μM concentrations of PFDA (Table IV). The remainder of experiments and statistical analyses will be performed in the next 2 months.

d. Significance

The enhancement of NK activity observed upon 30 day treatment with PFDA may be associated with anorixia as pair-fed controls exhibit similar changes and because no change in NK activity was observed 8 days following treatment. Changes in NK activity may be due to a relative increase in NK cells within the nonadherent spleen cell population, or may be due to increased activation of resident NK cells due to interferon gamma or IL-2 production. The slight increase of NK activity observed at 60 μM PFDA may not be statistically significant. Upon completion of these assays, this will be determined.

5. Effect of PFDA on antibody production 8 days and 30 days following PFDA treatment.

a. Rationale

Alterations in antigen-specific antibody production following PFDA treatment could compromise host defense against microbial infections.

b. Methods

Serum samples were harvested under general anesthesia by cardiac puncture. Samples were stored at -20°C until analysis. Samples were analyzed for KLH-specific IgG, IgM, and IgA by enzyme linked immunosorbent assay (ELISA).

c. Results

1. 8 day experiments.

KLH-specific IgG_{2a} levels at 8 days after PFDA treatment were decreased by 54% at a dose of 20 mg/kg, and by 69% at a dose of 50 mg/kg when compared to *ad libitum*-fed controls (Figure 7, top). KLH-specific IgM (Figure 8, top) and IgA levels (data not shown) were not significantly different from *ad libitum*-fed or pair-fed controls.

2. 30 day experiments.

KLH-specific IgG_{2a} levels (Figure 7, bottom), IgM levels (Figure 8, bottom), and IgA levels (data not shown) at 30 days after PFDA treatment were not significantly different from *ad libitum*-fed controls. A slight increase in IgG_{2a} production was observed at 50 mg/kg PFDA which was not statistically significant, but which is of interest.

d. Significance

The severe decrease in IgG_{2a} production observed at 8 days but not at 30 days following PFDA treatment corresponds with the changes in relative spleen weights, which are decreased at 8 days but not at 30 days following PFDA treatment. Other factors may be involved, however, including impaired T helper activity and thus decreased IL-2 production; or alternatively changes in macrophage production of IL-1 or PGE.

6. Effect of PFDA on IL-2 production and IL-2 receptor expression.

a. Rationale

Changes observed in cellular immunity, antigen-specific antibody production, or NK activity upon PFDA treatment may be attributable to alterations in IL-2 production, receptor expression, or responsiveness to IL-2.

b. Methods

1. IL-2 production.

Spleen cells (2×10^5 cells/well) from treated rats were incubated with or without 2 μg/ml Con A in 96 well round-bottom microtiter plates for 24 hours. Supernatants were harvested, filter sterilized, and frozen at -20°C until use. IL-2 containing supernatants from normal spleen cells treated in vitro with PFDA were also obtained. Samples will be assayed for IL-2 concentration by measuring proliferation of the IL-2 dependent cell line CTLL-2 in comparison to standards.

2. IL-2 receptor expression.

Spleen cell cultures were prepared as described above. Cells were pelleted and were washed with PBS. Cells were pelleted and monoclonal anti-rat interleukin II receptor (10 μl, Serotec) was added to the pellet at a 1:2 dilution. Cells were incubated at 4°C

for 30 minutes, washed 4 times with PBS, and fixed with 2% paraformaldahyde. Cells were analyzed by flow cytometry.

c. Results.

1. The IL-2 containing supernatants will be analyzed shortly.

2. Preliminary experiments on IL-2 receptor(R) expression demonstrate an increase in IL-2R on unstimulated and con A stimulated, PFDA-treated rats at a dose of 50 mg/kg 8 days (table V) and 30 days (Table VI) following PFDA treatment in vivo, and at 60 μM PFDA in vitro (Table VII). Further experiments needed to conclude this area of study will be performed in the next two months.

d. Significance.

The increase in IL-2R expression in unstimulated and stimulated PFDA-treated cells, without a corresponding increase in activation of T cells, leads to the hypothesis that these cells may have decreased signal transduction in response to IL-2 stimulation.

7. Effect of PFDA on spleen and thymic cell populations.

a. Rationale

An increase in the ratio of $CD8^+$(T suppressor/cytotoxic):$CD4^+$ (T helper cells) could be partially responsible for the suppression in the cellular and humoral responses described earlier. A decrease in the percentage of B cells in the spleen could contribute to the suppression of humoral immunity alone.

b. Methods

Spleen and thymic cells were isolated as described previously.

1. B cell percentages.

1×10^6 spleen cells were stained with monoclonal anti-rat LCA (10 μl, 1:100 dilution, present on B cells but not on thymocytes, Serotec) and were incubated 30 minutes at 4° C. Cells were washed 2 times with PBS and secondary antibody (FITC-conjugated goat anti-mouse IgG) was added (10 μl, 1:2 dilution) to the pellet. Cells were incubated 30 minutes at 4°C, and were washed 2 times with PBS. Cells were fixed in 2% paraformaldehyde and were analyzed by flow cytometry.

2. CD4, CD8 percentages.

1 x 10^6 spleen or thymus cells were pelleted and stained with monoclonal anti-rat CD4-FITC (10 μl, undiluted, Serotec) and monoclonal anti-rat CD8-phycoerythrin (10 μl, undiluted, Serotec) and were incubated 30 minutes at 4°C. Cells were washed 2 times with PBS, fixed in 2% paraformaldehyde, and analyzed by flow cytometry.

c. Results

1. <u>% B cells in the spleen</u>.

The experiments on B cell percentages in the spleen have been completed for 8 day and 30 day PFDA treatments. At 8 days following PFDA treatment, there are no significant differences in the percentage of B cells in PFDA treated rats when compared to *ad libitum*-fed control rats. At 30 days following PFDA treatment, there is an increase in the % of B cells in the 50 mg/kg PFDA-treated group which is not statistically significant.

2. <u>% $CD4^+$ and $CD8^+$ cells in the spleen and thymus</u>.

A few more experiments are required to complete this analysis. Preliminary data do not show dramatic changes in the percentages of these cells 8 days (Table 10) or 30 days (Table 11) following PFDA treatment in the spleen. The percentages of these cells in the thymus 8 days following PFDA treatment are altered at 50 mg/kg PFDA. There is a 10% increase in the % $CD4^+$ cells and a 20 % decrease in the percentage of double positive cells in the thymus (Table 12). Changes apparent at 30 days following PFDA treatment are less severe. There is a slight increase of 4% in $CD8^+$ cells and a corresponding decrease of 4% in double positive cells (Table 13).

d. Significance

The percentage of B cells is not altered in 8 day PFDA-treated rats, however, because the relative spleen weight is lower, this means that the total B cell number is decreased. The increase in the percentage of B cells along with no change in relative spleen weight observed 30 days following PFDA treatment at 50 mg/kg corresponds to the increase in IgG_{2a} observed at that dose in the 30 day experiments. This also explains increased response to B cell mitogens in nonadherent spleen cell populations. The changes in $CD4^+$ and $CD8^+$ populations in the spleen

and thymus require substantiation with further experiments.

8. Effect of PFDA on T cell and B cell capping.

a. Rationale

This assay will determine if there is a defect in the initial stages of cellular activation following receptor-ligand binding.

b. Methods

50 μl FITC-con A (200 μg/ml) or monoclonal anti-rat Ig-FITC (1:20 dilution) are added to pelleted spleen cells and are incubated at 4°C for 30 minutes. Cells are placed in a 37°C water bath and are removed and fixed in cold 2% paraformaldehyde at various time intervals. Capping is determined using fluorescence microscopy.

c. Results

The development of the method has been completed and experiments should be completed by July.

9. MHC II Expression

a. Rationale

These experiments will investigate if defective cellular and humoral immunity following PFDA treatment involves decreased MHC II expression and thus antigen presentation by macrophages and B cells to T cells.

b. Methods

Monoclonal anti-rat RT1B is diluted 1:10 and 10 μl is added to the cell pellet. Cells are incubated at 4°C for 30 minutes and are fixed in 2% paraformaldehyde.

c. Results

Titration of the antibody has been determined and experiments should be completed by July.

10. Phagocytosis and Oxidative Burst

a. Rationale

These experiments will determine if macrophage function against bacterial infection is normal following PFDA treatment.

b. Methods

Dichlorofluorescein diacetate and propidium iodide-stained staphylococcus aureus (50:1 bacteria:cell ratio) are incubated at 37°C with isolated and adherence purified peritoneal macrophages for 30 minutes on an orbital shaker. Cells are analyzed by flow cytometry.

c. Results

The methodology is still being improved. Experiments are scheduled to begin after final improvements have been implemented.

11. IL-1 Production

a. Rationale

If PFDA causes decreased IL-1 production by macrophages, this will result in reduced T cell activation and decreased IL-2 production and could be partially responsible for the changes in cellular and humoral immunity reported upon treatment with PFDA.

b. Methods

Peritoneal cells (1 x 10^6/ml) will be adherence purified and incubated with LPS at an optimal stimulatory concentration for 24 hours. Cell supernatants will be collected and stored at -20°C until assay. Supernatants will be analyzed either by bioassay with an IL-1 dependent cell line or by radioimmunoassay, to be determined after all samples have been collected.

12. PGE Production

a. Rationale

An increase in PGE production upon PFDA treatment may also alter IL-2 production, potentially through induction of T

suppressor cells.

b. Methods

Cells will be collected and treated as described in the previous section and supernatants will be collected and stored at -20°C until assay. Supernatants will be analyzed by a commercially available radioimmunoassay kit.

B. Hepatotoxicity of PFDA

1. Characterization of the kinetics of peroxisomal profiferation with PFDA treated hepatocytes in vivo and in vitro.

a. Rationale

Chemically unrelated compounds that induce peroxisome proliferation have been associated with hepatocellular carcinogenisis. Characterizing the kinectics of peroxisomal proliferation is the initial step in defining the mechanism of PFDA induced hepatocellular carcinogenisis. With these data we can elaborate by characterizing the kinetics of PFDA induced enzymes such as Cytochromes P-450 and enoyl Co-A hydratase.

b. Methods

Hepatocytes obtained from collagenase perfused livers and tissue culture (WB-F344) are treated with PFDA and then stained with dihydrorhodamine 123. Dihydrorhodamine 123 is a dye that is flourescent in the pressence of superoxide or hydrogen peroxide production. Peroxisomes are identified by intense areas of staining. With the use of the ACAS we hope to be able to measure the rate of peroxisome proliferation in PFDA-treated hepatocytes.

2. Production of polyclonal antibodies to Enoyl Co-A Hydratase in Chickens.

a. Rationale

Induction of enoyl Co-A hydratase during PFDA exposure is unique compared to the other known peroxisome proliferators. PFDA treated hepatocytes produce a substantially increased amount of enoyl Co-A hydratase. Production of polyclonal antibodies will allow fluorescent studies on the ACAS and flow cytometer. Chickens were used to prepare antisera because they provide a superior immunological responses to interspecies proteins. Chicken egg yolks is a good source of IgY polyclonal antibodies. This proceedure will enable us to collect the

antibodies for the lifespan of the chicken without the collection of blood.

b. Methods

Enoyl Co-A hydratase samples were obtained from Frank Witzman. Chickens were immunized with a mixture of enoyl Co-A hydratase in a acrylamide disk and Freunds complete adjuvant (50/50: w/w) for initial immunizations. Subsequent immunization used Freunds incomplete adjuvant and were performed at three week intervals. Eggs were collected daily and stored at 4°C until processed. The egg yolk antibodies are extracted using various concentrations of polyethylene glycol. Specificity of the polyclonal antibodies are determined using western blot analysis.

c. Results

Eggs from immunized chickens have been collected and extracted for immunoglobulin. We are presently obtaining a known enoyl Co-A hydratase sample for testing the specificity of the antibody.

3. Characterization of the kinetics of Cytochromes P-450 and Enoyl Co-A Hydratase induction after PFDA treatment.

a. Rationale

Hepatocellular enzymes, cytochrome P-450 and enoyl Co-A hydratase, are induced after PFDA treatment. These enzymes play an important role in omega oxidation of fatty acids. The omega oxidized fatty acids have been hypothesized to be a catalase for peroxisomal proliferation. Characterizing the kinetics of these enzymes might give us insight to the chronolgy of induction and the potential role of these enzymes in carcinogenesis.

b. Methods

Hepatocytes obtained from collagenase perfused livers and tissue culture (WB-F344) are treated with PFDA and then incubated with either fluorescent dyes for cytochrome P-450 or fluorescently labeled antibodies to enoyl Co-A hydratase. With the use of the ACAS we can observe the rate of enzyme induction of the same cell over time.

4. Measurement of hepatocellular IgA receptors.

a. Rationale

A well recognized mechanism for clearence of orally injested antigens is via hepatic clearance of polymophic IgA-antigen immune complexes. Many hepatic diseases are associated with elevated serum levels of polymorphic IgA either free

or complexed with antigen, suggesting decreased hepatic clearence. It has been reported that TCDD increased serum IgA levels and decreased bile IgA levels in treated rats, which was attributed to decreased clearance (Moran et al.,1986). Although PFDA does not significantly alter serum IgA levels 8 days or 30 days following treatment, we will determine if PFDA alters the expression of hepatocellular IgA receptors.

b. Methods

Hepatocytes obtained from collagenase perfused livers and tissue culture (WB-F344) will be incubated with commercially available rabbit anti-rat IgA secretory piece, washed and incubated with FITC-conjugated goat anti-rabbit immunoglobin.

III. LIST OF PUBLICATIONS

A. D.L. Nelson, D.E. Frazier, Jr., J.E. Ericson, M.J. Tarr, and L.E. Mathes. 1992. The effects of Perfluorodecanoic Acid (PFDA) on humoral, cellular, and innate immunity in Fischer 344 rats. J. Immunopharm. Immunotox. (submitted)

B. D.L. Nelson, M.J. Tarr, and L.E. Mathes. 1992. Alterations in immune function associated with in vitro PFDA treatment. (in preparation)

C. D.L. Nelson, D.E. Frazier, Jr., J.E. Ericson, M.J. Tarr, and L.E. Mathes. 1992. Mechanisms of PFDA-induced immunomodulation. (in preparation)

IV. PROFESSIONAL PERSONNEL ASSOCIATED WITH RESEARCH EFFORT

A. Melinda J. Tarr, D.V.M., Ph.D., Principal Investigator.

Dr. Tarr is responsible for the overall administration of the project.

B. Lawrence E. Mathes, Ph.D., Co-Principal Investigator.

Dr. Mathes replaced Dr. Krakowka and is responsible for day to day supervision of Ms. Nelson and Mr. Pollman, as well as administration of the hepatocyte studies.

C. Donald E. Frazier, Jr., Research Associate.

Dr. Frazier left in August, 1991. Until that time, he was responsible for coordination of experimental procedures, graduate student training, data collection and report writing.

D. Debra L. Nelson, B.S., M.S., Graduate Research Associate.

Ms. Nelson is a graduate student working towards her Ph.D. Ms. Nelson is fully supported by the grant and is working on the immunotoxicological studies.

E. Mark K. Pollman, B.S., M.S., Research Associate.

Mr. Pollman was hired in December 1991, and is responsible for the hepatocyte studies. He is fully supported by the grant.

V. ABSTRACTS AND PRESENTATIONS AT CONFERENCES

1. Nelson, D.L., D.E. Frazier, Jr., J.E. Ericson, and M.J. Tarr. 1992. The effect of perfluorodecanoic acid on natural killer cell activity in Fischer 344 rats. The Toxicologist. 12(1):49.

2. Nelson, D.L., D.E. Frazier, Jr., and M.J. Tarr. 1992. The effect of perfluorodecanoic acid on antibody production in Fischer 344 rats. FASEB Journal. 6(5):A1875.

3. Nelson, D.L., D.E. Frazier, Jr., and M.J. Tarr. 1991. Evaluation of histopathologic changes and mitogen-induced proliferative response of lymphoid tissues isolated from perfluorodecanoic acid. 19th Conference on Toxicology, Dayton, Ohio.

VI. DISCOVERIES, INVENTIONS, OR PATENT DISCLOSURES

None

VII. OTHER STATEMENTS OF ADDITIONAL INSIGHT TO PROGRAM MANAGER

We will be completing the proposed immunotoxicological studies within the next 4 months and will be focusing on the hepatocyte studies in collaboration with Wright-Patterson Air Force Base (WPAFB). Under advisement of WPAFB we have altered our hepatocellular objectives as proposed in the grant. Production of polyclonal antibodies to PFDA induced enzymes and examining hepatocellular IgA receptors are still being pursued. Research will be performed on the kinetics of peroxisome proliferation and the enzymes that are induced after PFDA exposure.

Further immunotoxicological studies have been suggested by our findings and these include:

1. Analysis of % NK cells in spleens of control versus PFDA-treated rats.
2. Analysis of serum levels of IFNγ in control and PFDA-treated rats (known NK cell activator).
3. Analysis of serum levels of TNFα (known to cause cachexia and may suppress antibody production).
4. Analysis of signal transduction in response to IL-2.
5. Analysis of mixed lymphocyte response to confirm alterations in cellular immunity.

These studies and others may be included in future proposals.

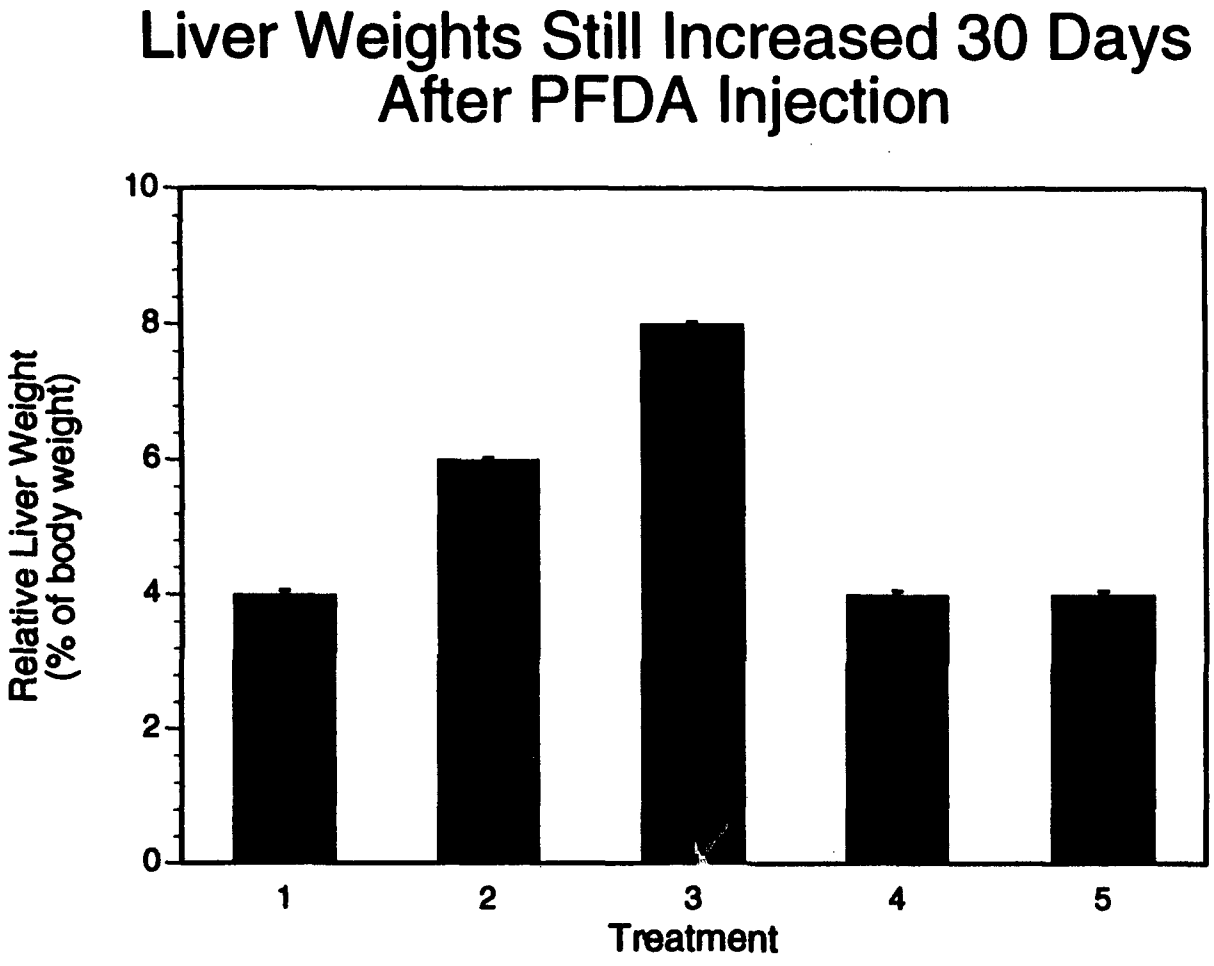

Figure 1. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Rats were sacrificed under general anesthesia, and organs were asceptically removed and weighed. Shown here is relative liver weight (as a percentage of body weight) versus treatment. Treatment 1 = *ad libitum*-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

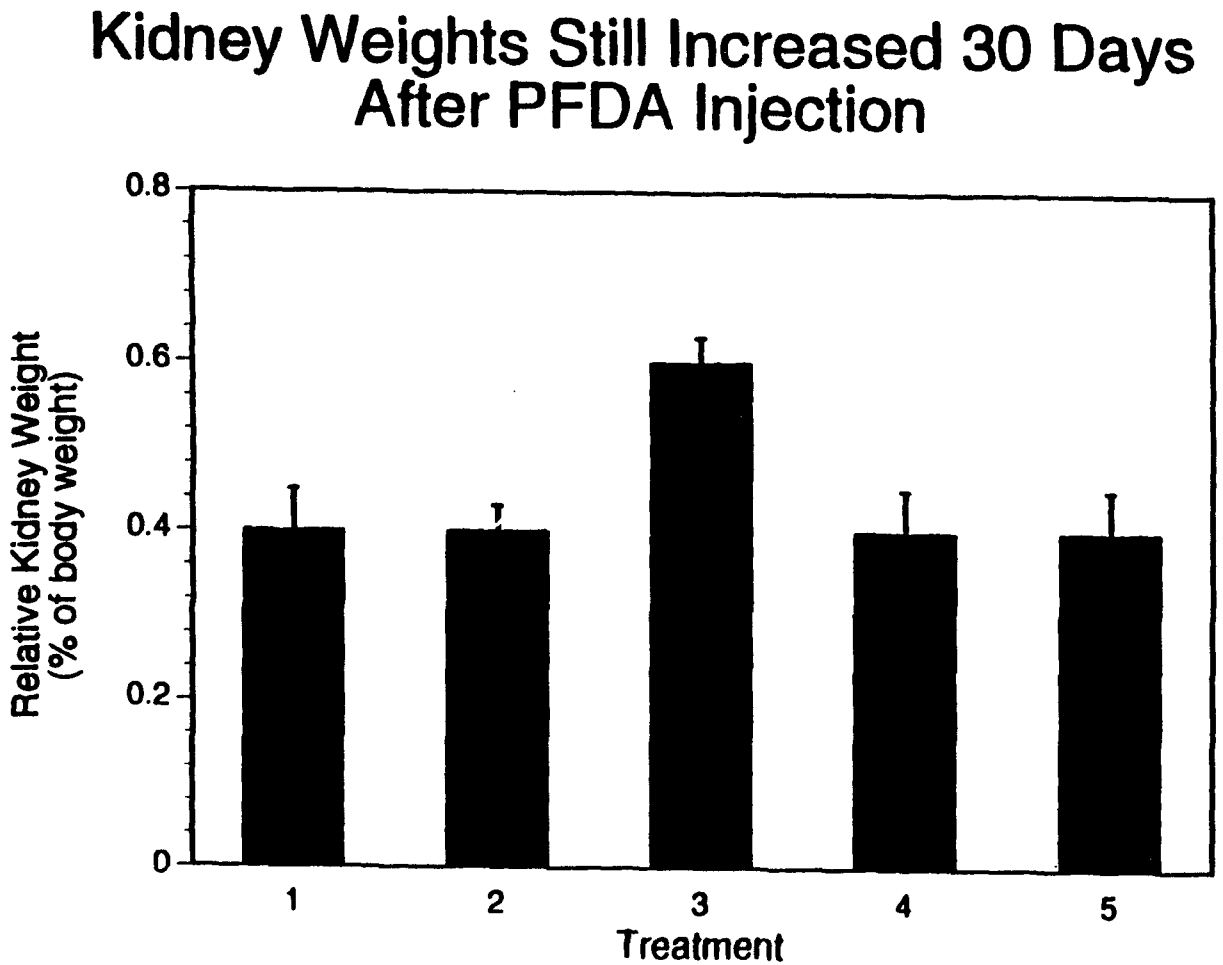

Figure 2. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Rats were sacrificed under general anesthesia, and organs were asceptically removed and weighed. Shown here is relative kidney weight (as a percentage of body weight) versus treatment. Treatment 1 = *ad libitum*-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

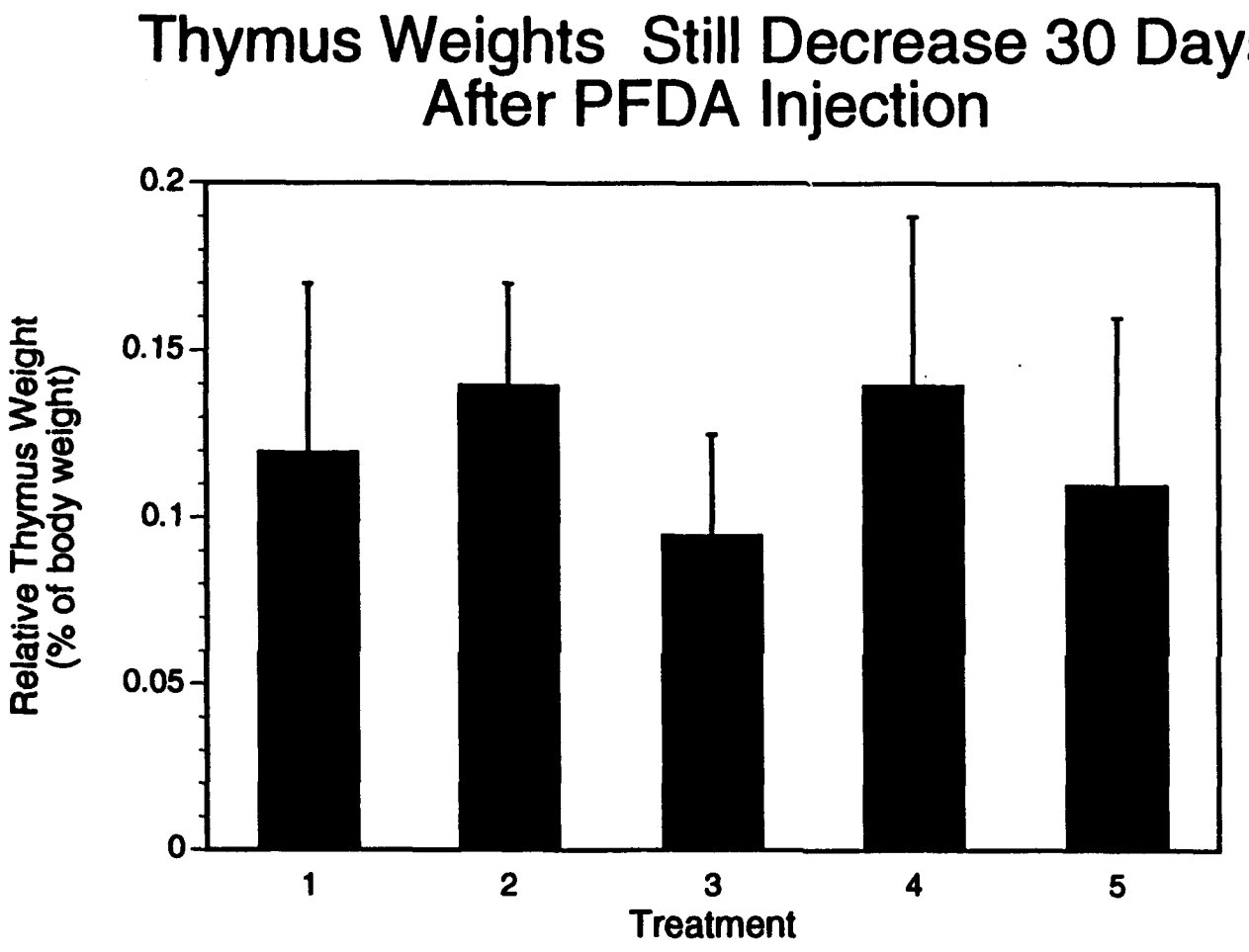

Figure 3. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Rats were sacrificed under general anesthesia, and organs were asceptically removed and weighed. Shown here is relative thymus weight (as a percentage of body weight) versus treatment. Treatment 1 = *ad libitum*-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

Spleen Weights Similar 30 Days After PFDA Injection

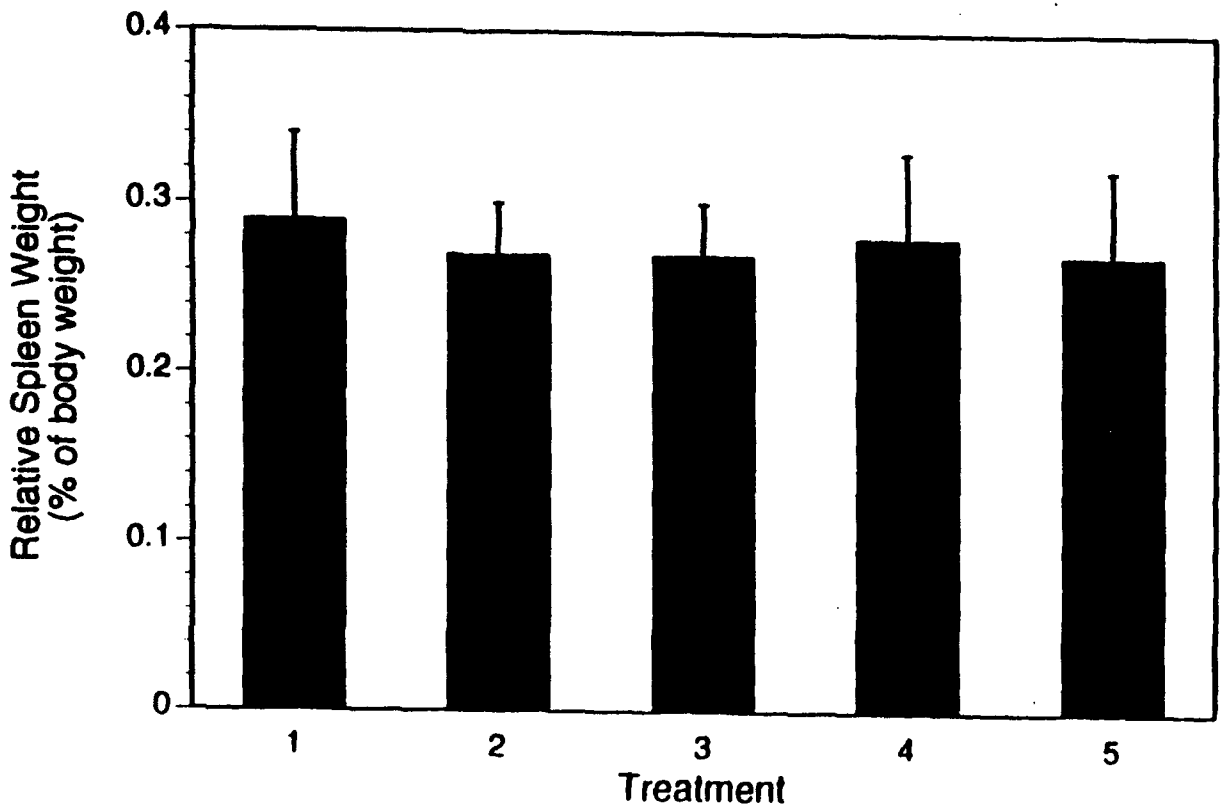

Figure 4. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Rats were sacrificed under general anesthesia, and organs were asceptically removed and weighed. Shown here is relative spleen weight (as a percentage of body weight) versus treatment. Treatment 1 = *ad libitum*-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

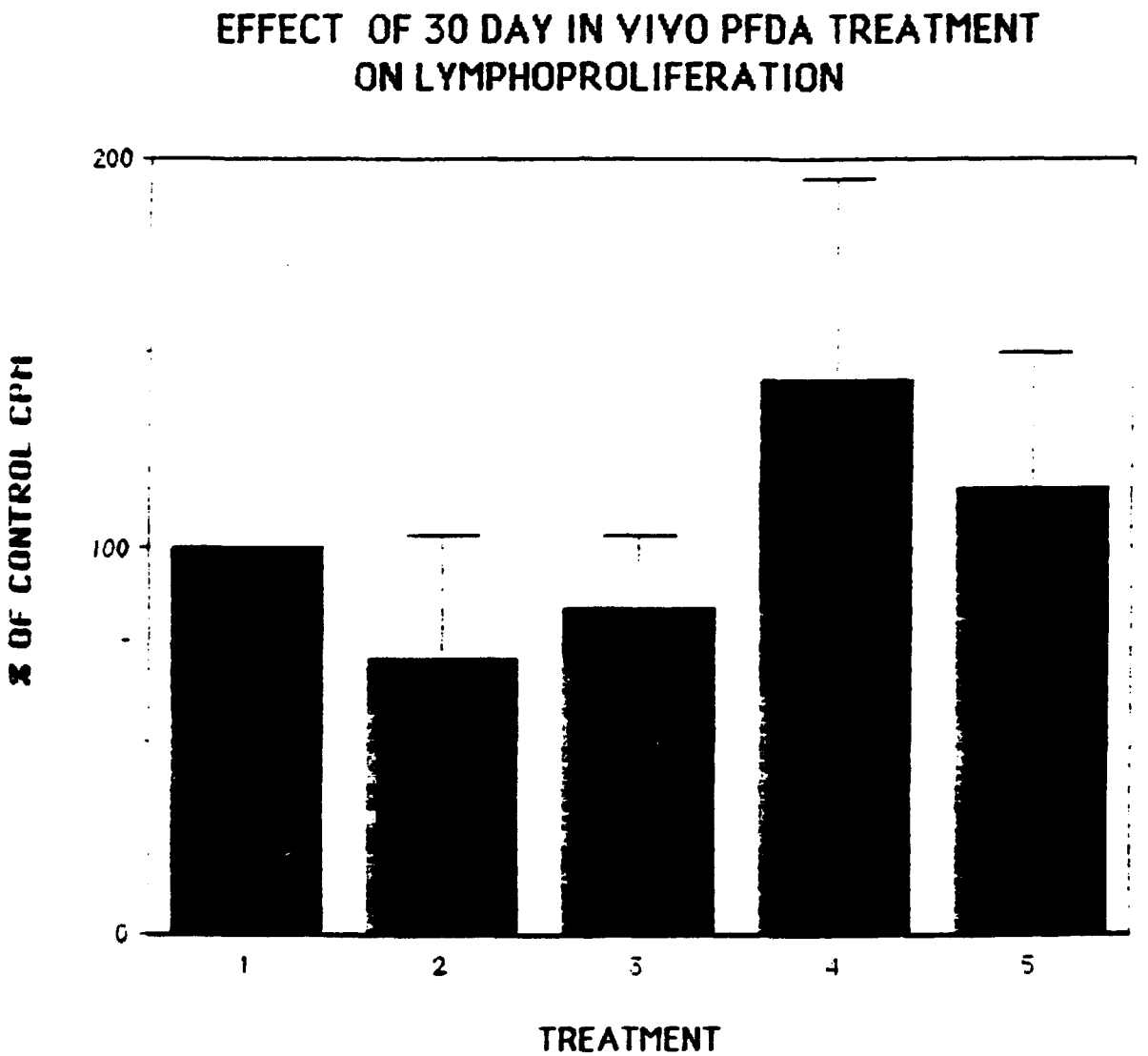

Figure 5. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Rats were sacrificed under general anesthesia, spleens were asceptically removed and mononuclear cell suspensions were prepared. Cells were incubated ± con A for 72 hours. In the last 6 hours of incubation, ^{3}H-thymidine was added. Incorporation of ^{3}H-thymidine was determined by liquid scintillation counting. The data are represented as the % of ad libitum-fed control counts per minute (CPM) versus treatment, n = 3. Treatment 1 = ad libitum-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

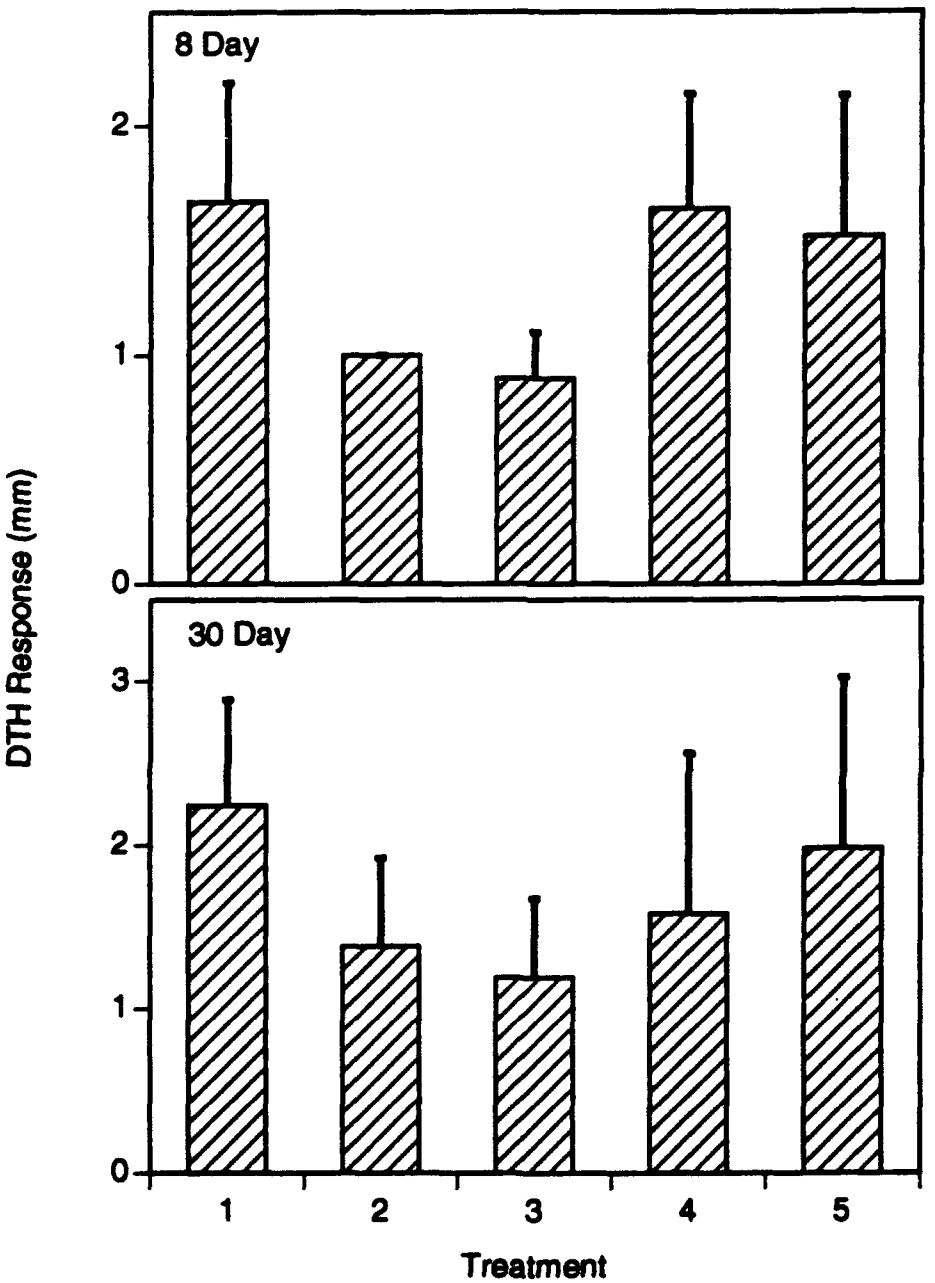

Figure 6. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 8 days (top) or 30 days (bottom) prior to sacrifice. These rats were immunized with keyhole limpet hemocyanin 14 days and 7 days prior to sacrifice. Immunized rats received an injection of heat-aggregated KLH in one footpad and a normal saline injection in the other footpad 24 hours prior to sacrifice. Footpad swelling was measured with vernier calipers. The DTH response is defined as the difference between swelling in the KLH-treated footpad and the normal saline-treated footpad. These data represent the DTH response (mm) from 5 representative treated animals. Treatment 1 = *ad libitum*-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

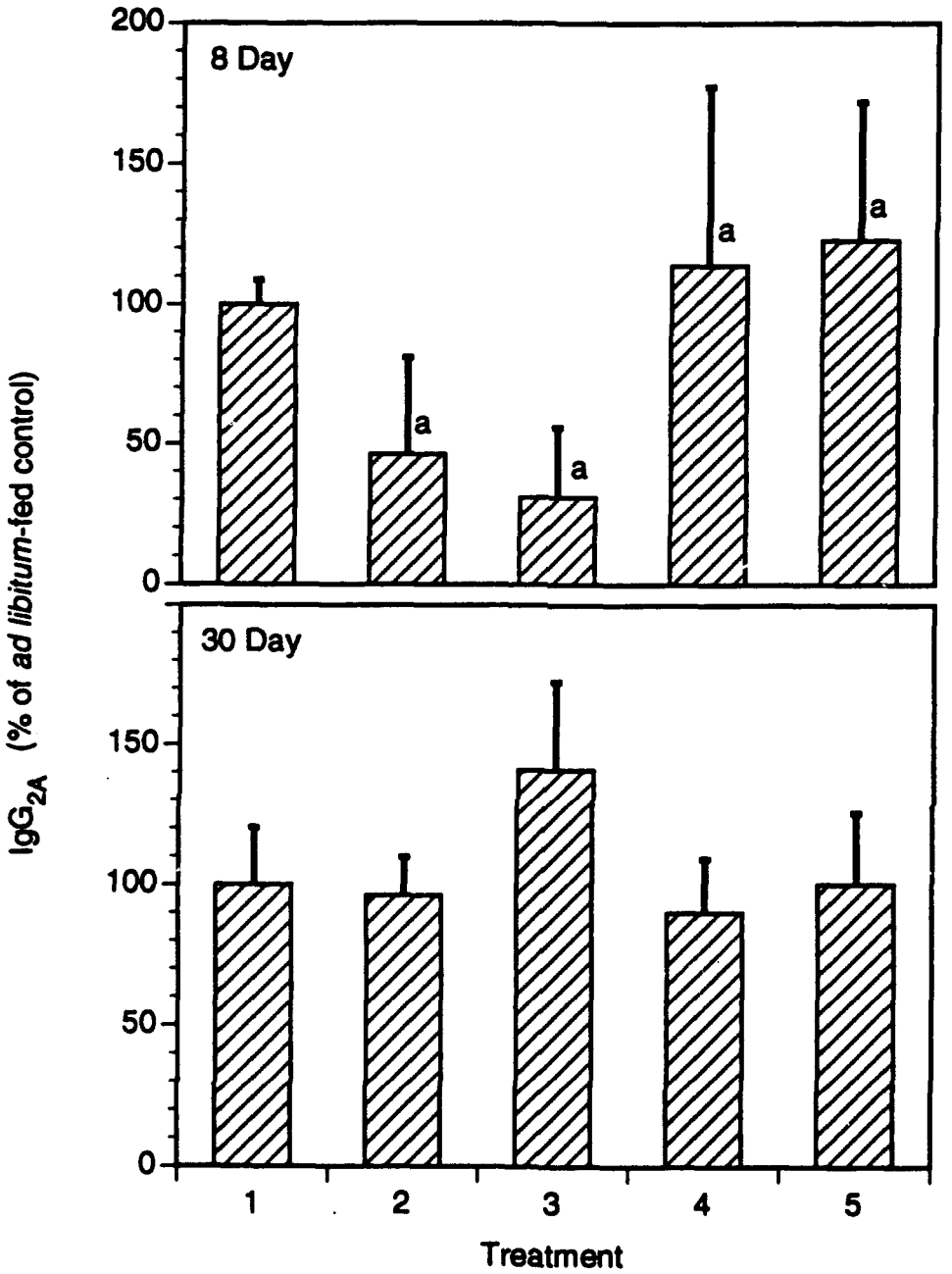

Figure 7. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 8 days (top) or 30 days (bottom) prior to sacrifice. Blood samples were collected by cardiac puncture under general anesthesia. Serum IgG_{2a} levels were determined by ELISA. IgG_{2a} levels are expressed as a percentage of the mean ad libitum-fed control level. Mean ± s.d. is shown, (n=5). Treatment 1 = *ad libitum*-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg. (a). Significantly different from the ad libitum-fed control by Tukey's studentized range test ($p < 0.05$).

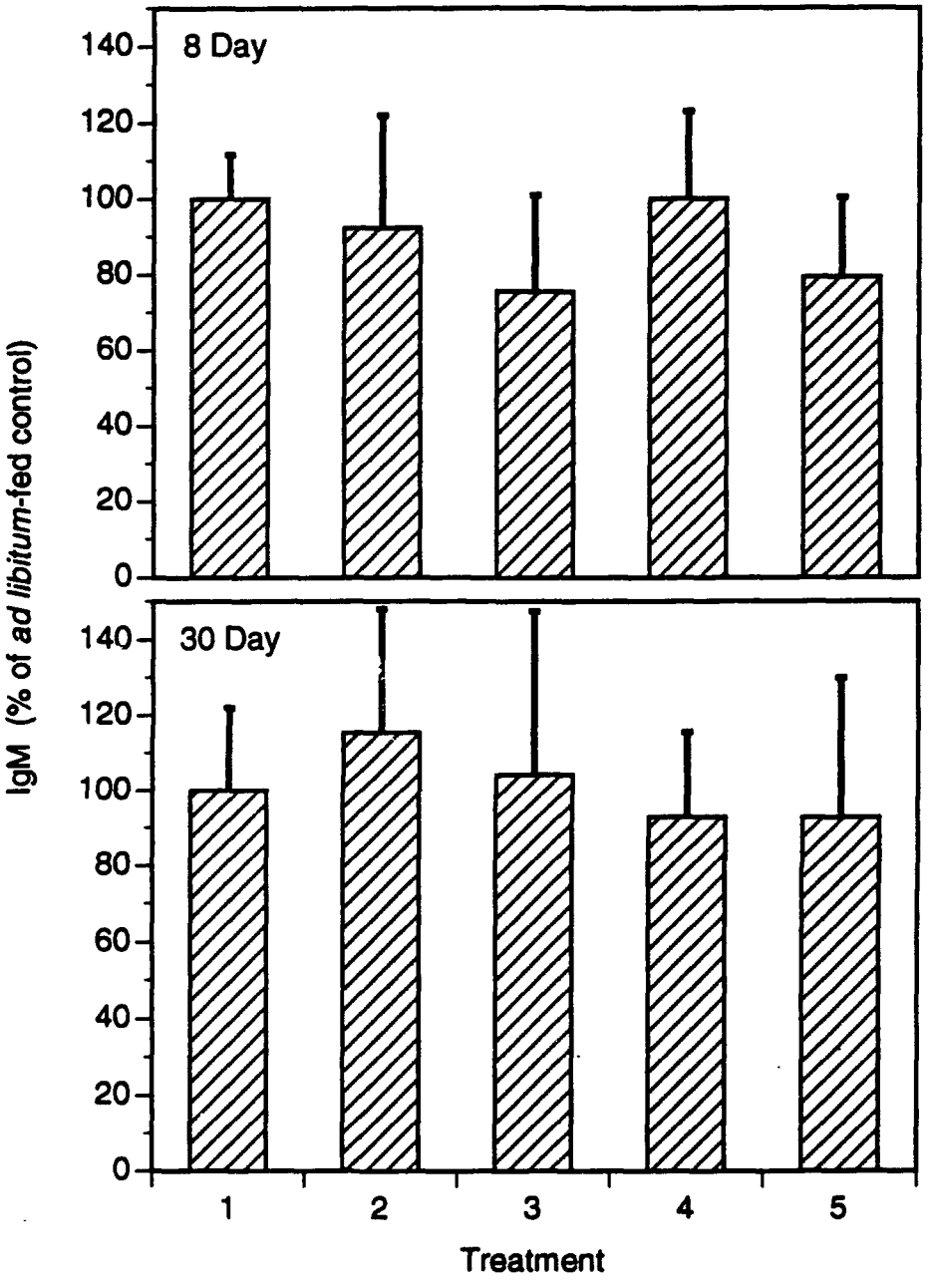

Figure 8. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 8 days (top) or 30 days (bottom) prior to sacrifice. Blood samples were collected by cardiac puncture under general anesthesia. Serum IgM levels were determined by ELISA. IgM levels are expressed as a percentage of the mean *ad libitum*-fed control level. Mean ± s.d. is shown, (n=5). Treatment 1 = *ad libitum*-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

Effect of in vitro PFDA treatment on lymphoproliferation

Treatment	Average CPM	% Viability
150 μM PFDA	1331 $\pm$ 1646	65
100 μM PFDA	48734 $\pm$ 20249	79
80 μM PFDA	52198 $\pm$ 21867	92
60 μM PFDA	45927 $\pm$ 21099	84
40 μM PFDA	64581 $\pm$ 11424	88
20 μM PFDA	98436 $\pm$ 13929	87
0.1% Propylene Glycol	150686 $\pm$ 23382	87
0% propylene glycol/PFDA	142400 $\pm$ 24084	80

Table I. Untreated Fischer 344 rats were sacrificed under general anesthesia, spleens were asceptically removed and mononuclear cell suspensions were prepared. Cells were incubated with various concentrations PFDA (0 - 150 μM) $\pm$ con A for 72 hours. In the last 6 hours of incubation, ^{3}H-thymidine was added. Incorporation of ^{3}H-thymidine was determined by liquid scintillation counting. The data are represented as the average counts per minute (CPM) versus treatment, and % viability at each treatment is indicated. Shown is one representative experiment. Three out of 4 experiments demonstrated suppression of Con A responses upon PFDA treatment.

Effect of 8 day in vivo PFDA Treatment on NK cytotoxicity

	% Cytotoxicity		
Treatment	100:1 E:T Ratio	50:1 E:T Ratio	25:1 E:T Ratio
Ad libitum-fed	28.3 ± 9.2	19.9 ± 7.4	13.9 ± 3.8
PFDA (20 mg/kg)	29.8 ± 10.1	19.9 ± 6.2	13.9 ± 4.1
PFDA (50 mg/kg)	27.9 ± 12.9	21.3 ± 8.3	14.1 ± 6.2
Pair-fed (to 20 mg/kg)	29.1 ± 10.0	20.3 ± 5.0	13.0 ± 5.6
Pair-fed (to 50 mg/kg)	33.1 ± 12.8	25.4 ± 11.4 (a)	17.3 ± 8.7 (a)

Table II. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 8 days prior to sacrifice. Nonadherent spleen cells were cultured with PKH-stained YAC-1 target cells at various effector:target (E:T) ratios for 4 hours at 37°C. Percent specific cytotoxicity was determined by flow cytometry following propidium iodide incorporation. Experiments were performed in triplicate and mean ± s.d. for 4 experiments is shown. (a). significantly different from *ad libitum*-fed control by Tukey's studentized range test. Treatment 1 = ad libitum-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

Effect of 30 day in vivo PFDA Treatment on NK cytotoxicity

Treatment	% Cytotoxicity		
	100:1 E:T Ratio	50:1 E:T Ratio	25:1 E:T Ratio
Ad libitum-fed	27.8 ± 7.1	18.4 ± 6.9	12.3 ± 4.2
PFDA (20 mg/kg)	29.8 ± 13.0	21.0 ± 12.5	12.6 ± 8.1
PFDA (50 mg/kg)	34.3 ± 8.7 (a)	20.8 ± 6.2	14.8 ± 5.6 (a)
Pair-fed (to 20 mg/kg)	34.3 ± 13.8 (a)	22.7 ± 11.5 (a)	15.8 ± 7.4 (a)
Pair-fed (to 50 mg/kg)	33.1 ± 17.1 (a)	21.6 ± 12.1 (a)	15.7 ± 8.5 (a)

Table III. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Nonadherent spleen cells were cultured with PKH-stained YAC-1 target cells at various effector:target (E:T) ratios for 4 hours at 37°C. Percent specific cytotoxicity was determined by flow cytometry following propidium iodide incorporation. Experiments were performed in triplicate and mean ± s.d. for 4 experiments is shown. (a). significantly different from *ad libitum*-fed control by Tukey's studentized range test. Treatment 1 = ad libitum-fed control; Treatment 2 = PFDA, 20 mg/kg; Treatment 3 = PFDA, 50 mg/kg; Treatment 4 = Pair-fed to 20 mg/kg; Treatment 5 = Pair-fed to 50 mg/kg.

Effect of in vitro PFDA Treatment on NK cytotoxicity

	% Cytotoxicity		
Treatment	100:1 E:T Ratio	50:1 E:T Ratio	25:1 E:T Ratio
150 μM PFDA	39.8 ± 5.0	32.8 ± 4.9	24.0 ± 4.2
100 μM PFDA	43.8 ± 9.0	31.4 ± 5.9	23.3 ± 5.6
80 μM PFDA	42.4 ± 9.2	31.2 ± 5.8	22.6 ± 3.3
60 μM PFDA	46.9 ± 7.9	37.9 ± 6.0	30.3 ± 6.6
40 μM PFDA	45.3 ± 3.0	35.0 ± 3.7	26.3 ± 1.8
20 μM PFDA	40.4 ± 7.1	32.8 ± 8.3	21.8 ± 5.3
0.1% Propylene Glycol	42.0 ± 8.2	33.0 ± 6.5	22.2 ± 5.7
0% propylene glycol, 0% PFDA	44.5 ± 9.8	34.1 ± 7.5	22.8 ± 6.2

Table IV. Nonadherent spleen cells obtained from untreated Fischer 344 rats were cultured with PKH-stained YAC-1 target cells at various effector:target (E:T) ratios for 4 hours at 37°C. PFDA (0 - 150μM) was present for the duration of the incubation period. Percent specific cytotoxicity was determined by flow cytometry following propidium iodide incorporation. Experiments were performed in triplicate and mean ± s.d. for 3 experiments is shown.

Effect of 8 day in vivo PFDA treatment on IL-2R expression

Treatment	% IL-2R$^+$ Cells (unstimulated)	% IL-2R$^+$ Cells + Con A
Ad libitum-fed control	24.3	79.7
PFDA 20 mg/kg	22.0	82.0
PFDA 50 mg/kg	37.7	91.2
Pair-fed (to 20 mg/kg)	45.3	91.8
Pair-fed (to 50 mg/kg)	42.2	86.5

Table V. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 8 days prior to sacrifice. Spleens were isolated and mononuclear cell suspensions were prepared. Cells were incubated ± Con A for 24 hours, cells were washed and then stained with monoclonal anti-rat IL-2 receptor. % positive cells was determined by flow cytometry. These data are from a preliminary experiment, performed in duplicate.

Effect of 30 day in vivo PFDA treatment on IL-2R expression

Treatment	% IL-2R$^+$ Cells (unstimulated)	% IL-2R$^+$ Cells + Con A
Ad libitum-fed control	15.6 ± 3.5	65.0 ± 5.4
PFDA 20 mg/kg	21.9 ± 14.7	68.19 ± 9.1
PFDA 50 mg/kg	49.0 ± 13.7	73.6 ± 2.5
Pair-fed (to 20 mg/kg)	32.0 ± 17.3	60.8 ± 4.0
Pair-fed (to 50 mg/kg)	34.6 ± 31.4	66.4 ± 9.8

Table VI. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Spleens were isolated and mononuclear cell suspensions were prepared. Cells were incubated ± Con A for 24 hours, cells were washed and then stained with monoclonal anti-rat IL-2 receptor. The percentage of positive cells was determined by flow cytometry. These data represent the mean % IL-2R$^+$ cells ± s.d. for 2 experiments, performed in duplicate.

Effect of in vitro PFDA treatment on IL-2 Receptor Expression

Treatment	% IL-2 $^{+}$ cells (unstimulated)	% IL-2 $^{+}$ cells (stimulated with con A)
150 μM PFDA	23 $\pm$ 11	65.5 $\pm$ 13
100 μM PFDA	20 $\pm$ 6.5	62 $\pm$ 16
80 μM PFDA	18 $\pm$ 6.0	66 $\pm$ 14
60 μM PFDA	26 $\pm$ 9.0	70 $\pm$ 13
40 μM PFDA	18.7 $\pm$ 9.2	66 $\pm$ 18
20 μM PFDA	19.5 $\pm$ 6.1	64 $\pm$ 15
0.1% Propylene glycol	19.0 $\pm$ 5.0	64 $\pm$ 15
0% Propylene glycol/PFDA	20.0 $\pm$ 8.1	65 $\pm$ 17

Table VII. Spleens from untreated Fischer 344 rats were isolated and mononuclear cell suspensions were prepared. Cells were incubated with variuos concentrations PFDA (0 - 150 μM) $\pm$ Con A for 24 hours, cells were washed and then stained with monoclonal anti-rat IL-2 receptor. The percentage of positive cells was determined by flow cytometry. These data represent the mean % IL-2R^{+} cells $\pm$ s.d. for 2 experiments, performed in duplicate.

Effect of 8 day in vivo PFDA exposure on B cell Populations in the spleen

Treatment Group	% B cells
Ad libitum-fed Control	28.2 ± 6.6
PFDA (20 mg/kg)	30.4 ± 7.1
PFDA (50 mg/kg)	28.7 ± 7.4
Pair-fed (to 20 mg/kg)	33.5 ± 8.1
Pair-fed (to 50 mg/kg)	22.8 ± 5.4

Table VIII. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 8 days prior to sacrifice. Spleens were isolated and mononuclear cell suspensions were prepared. Cells were incubated with anti-rat LCA (present on B, not T cells). The percentage of positive cells was determined by flow cytometry. These data represent the mean % B cells ± s.d. for 4 experiments, performed in duplicate.

Effect of 30 day in vivo PFDA exposure on B cell Populations in the spleen

Treatment Group	% B cells
Ad libitum-fed Control	25.4 ± 6.7
PFDA (20 mg/kg)	22.6 ± 7.7
PFDA (50 mg/kg)	31.7 ± 7.7
Pair-fed (to 20 mg/kg)	18.8 ± 7.8
Pair-fed (to 50 mg/kg)	22.3 ± 5.3

Table IX. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Spleens were isolated and mononuclear cell suspensions were prepared. Cells were incubated with anti-rat LCA (present on B, not T cells). The percentage of positive cells was determined by flow cytometry. These data represent the mean % B cells ± s.d. for 4 experiments, performed in duplicate.

Effect of 8 day in vivo PFDA exposure on T lymphocytes in the spleen

Treatment Group	% $CD4^+$ Cells	% $CD8^+$ Cells	% Double Positive Cells
Ad libitum-fed control	32.8 ± 6.5	34.2 ± 6.8	7.5 ± 6.5
PFDA (20 mg/kg)	34.6 ± 4.0	35.5 ± 5.5	7.3 ± 5.6
PFDA (50 mg/kg)	34.1 ± 5.4	28.7 ± 5.2	10.7 ± 6.1
Pair-fed (to 20 mg/kg)	33.9 ± 4.4	29.5 ± 4.3	8.8 ± 6.0
Pair-fed (to 50 mg/kg)	35.1 ± 8.1	31.2 ± 4.4	6.6 ± 4.7

Table X. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 8 days prior to sacrifice. Spleens were isolated and mononuclear cell suspensions were prepared. Cells were incubated with anti-rat CD4-FITC and CD8-phycoerythrin. The percentage of positive cells was determined by flow cytometry. These data represent the mean % $CD4^+$, $CD8^+$, and double positive cells ± s.d. for 2 experiments, performed in duplicate.

Effect of 30 day in vivo PFDA exposure on T lymphocytes in the spleen

Treatment Group	% $CD4^+$ Cells	% $CD8^+$ Cells	% Double Positive Cells
Ad libitum-fed control	28.7 ± 6.0	29.7 ± 4.3	5.5 ± 3.9
PFDA (20 mg/kg)	27.3 ± 4.2	34.7 ± 6.3	3.6 ± 1.9
PFDA (50 mg/kg)	29.7 ± 2.6	32.7 ± 4.6	4.6 ± 2.4
Pair-fed (to 20 mg/kg)	30.9 ± 7.4	39.2 ± 7.6	3.5 ± 0.7
Pair-fed (to 50 mg/kg)	28.2 ± 5.1	32.0 ± 9.1	4.2 ± 1.2

Table XI. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Spleens were isolated and mononuclear cell suspensions were prepared. Cells were incubated with anti-rat CD4-FITC and CD8-phycoerythrin. The percentage of positive cells was determined by flow cytometry. These data represent the mean % $CD4^+$, $CD8^+$, and double positive cells ± s.d. for 2 experiments, performed in duplicate.

Effect of 8 day in vivo PFDA exposure on T lymphocytes in the thymus

Treatment Group	% $CD4^+$ Cells	% $CD8^+$ Cells	% DP Cells	% DN Cells
Ad libitum-fed control	20.0 ± 4.0	14.1 ± 3.8	64.1 ± 5.4	4.6
PFDA (20 mg/kg)	16.4 ± 2.5	13.9 ± 1.7	66.7 ± 2.1	2.9
PFDA (50 mg/kg)	30.2 ± 7.4	19.5 ± 3.3	43.7 ± 10	6.7
Pair-fed (to 20 mg/kg)	16.0 ± 2.0	13.8 ± 1.2	66.9 ± 4.4	3.3
Pair-fed (to 50 mg/kg)	17.3 ± 2.1	14.3 ± 1.9	64.6 ± 2.7	3.8

Table XII. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 8 days prior to sacrifice. Thymuses were isolated and mononuclear cell suspensions were prepared. Cells were incubated with anti-rat CD4-FITC and CD8-phycoerythrin. The percentage of positive cells was determined by flow cytometry. These data represent the mean % $CD4^+$· $CD8^+$, double positive and double negative cells ± s.d. for 2 experiments, performed in duplicate.

Effect of 30 day in vivo PFDA exposure on T lymphocytes in the thymus

Treatment Group	% $CD4^+$ Cells	% $CD8^+$ Cells	% DP Cells	% DN Cells
Ad libitum-fed control	7.3 ± 3.2	15.5 ± 7.1	68.1 ± 6.3	6.3
PFDA (20 mg/kg)	8.2 ± 4.5	17.3 ± 5.8	65.6 ± 2.9	6.9
PFDA (50 mg/kg)	7.7 ± 3.0	19.3 ± 10	64.3 ± 7.8	6.6
Pair-fed (to 20 mg/kg)	8.5 ± 2.4	18.2 ± 8.6	64.4 ± 9.1	7.0
Pair-fed (to 50 mg/kg)	7.6 ± 3.2	17.1 ± 8.9	66.6 ± 8.5	6.9

Table XIII. Fischer 344 rats received a single intraperitoneal injection of PFDA (20 mg/kg or 50 mg/kg) 30 days prior to sacrifice. Thymuses were isolated and mononuclear cell suspensions were prepared. Cells were incubated with anti-rat CD4-FITC and CD8-phycoerythrin. The percentage of positive cells was determined by flow cytometry. These data represent the mean % $CD4^+$, $CD8^+$, double positive and double negative cells ± s.d. for 2 experiments, performed in duplicate.