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Published online: 28 Sep 2008.

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THE EFFECTS OF PERFLUORODECANOIC ACID (PFDA) ON HUMORAL, CELLULAR, AND INNATE IMMUNITY IN FISCHER 344 RATS

Debra L. Nelson*, Donald E. Frazier, Jr., Julie E. Ericson, Melinda J. Tarr and Lawrence E. Mathes.
Department of Veterinary Pathobiology
The Ohio State University, Columbus, Ohio 43210

ABSTRACT

The in vivo effects of perfluorodecanoic acid (PFDA) exposure on antibody production, delayed type hypersensitivity (DTH), and natural killer (NK) cell activity were determined. Fischer 344 rats were injected with PFDA (20 mg/kg or 50 mg/kg, 8 days or 30 days prior to sacrifice) and were immunized with keyhole limpet hemocyanin (KLH). Pair-fed and ad libitum-fed control rats were included to evaluate effects of PFDA-induced anorexia. KLH-specific IgG_{2a} production was significantly decreased ($p < 0.05$) in PFDA-treated rats when compared to ad libitum-fed and pair-fed controls at 8 days but not at 30 days following PFDA treatment. The DTH response of PFDA-treated rats was decreased 8 days and 30 days after PFDA treatment when compared to ad libitum-fed and pair-fed controls, however, the decrease was not statistically significant. NK activity 30 days after PFDA treatment was significantly elevated ($p < 0.05$) when compared to ad libitum-fed controls, but pair-fed controls had similarly elevated NK activity. NK activity at 8 days after PFDA treatment was not significantly altered. In conclusion, PFDA has been demonstrated to have immunomodulatory effects, some of which may be associated with drug-induced anorexia.

INTRODUCTION

Exposure to perfluorinated carboxylic acids, including perfluorodecanoic acid (PFDA[1]) occurs primarily through the use of commercial wetting agents and flame retardants (1). Perfluorinated carboxylic acids were originally considered to be inert, but subsequent analyses have demonstrated substantial toxicity. PFDA is eliminated slowly from the body ($t_{1/2}$ = 23 days for Sprague Dawley rats), and therefore, longterm effects may occur from a single injection (2). Studies by Langley and Pilcher (3), showed that PFDA (75 mg/kg) causes bradycardia, hypothermia, and decreased serum thyroid hormone levels in Wistar rats. Andersen et al. (4), demonstrated that PFDA (90 mg/kg) causes anorexia, loss of body weight, and death within 2-3 weeks in Fischer 344 rats. Pathological findings included thymic atrophy and hemorrhage, bone marrow depletion, hepatic necrosis, bile duct proliferation, gastritis, and necrosis of the testes. In Fischer 344 rats, PFDA (50 mg/kg), causes enlargement of the liver due to persistent cellular swelling (5), peroxisomal proliferation, and hyperplasia (6,7,8).

Information available on the immunotoxicity of PFDA is limited. Hayek et al. (9), found that PFDA (35 mg/kg) in vivo, did not alter the responsiveness of spleen cells isolated at 8 days and 16 days post-injection to the mitogens Concanavalin A, phytohemagglutinin, pokeweed mitogen, and lipopolysaccharide. Levitt and Liss (10), found that PFDA (<4 mM, in vitro) did not alter immunoglobulin production by the F4, Hururtz, and HPCM2 B cell lines, nor did it alter capping of surface IgM on these cells (11).

This study further evaluates the immunotoxicity of PFDA in vivo. The effects of PFDA (20 mg/kg and 50 mg/kg) on humoral immunity, cellular immunity, and natural surveillance in Fischer 344 rats at 8 days and 30 days following PFDA injection are determined.

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1. Abbreviations: PFDA, perfluorodecanoic acid; KLH, keyhole limpet hemocyanin; OPD, orthophenylenediamine; NK, natural killer; PI, propidium iodide; DTH, delayed type hypersensitivity; E:T, effector:target.

METHODS AND MATERIALS

Animals

Male Fischer 344 rats, 7-9 weeks of age, were purchased from Harlan Sprague Dawley. Rats were acclimatized for 1 week at the animal resources facility prior to beginning the experiments.

Materials

PFDA was purchased from the Aldrich Chemical Company (Milwaukee, WI). KLH, used for immunization was purchased from ICN Biomedicals (Costa Mesa, CA). Polyclonal rabbit anti-rat immunoglobulin antibodies were obtained from Bioproducts for Science, Inc. (Indianapolis, IN). Immunoglobulin standards and orthophenylenediamine (OPD) were purchased from Zymed (San Francisco, CA). YAC-1 mouse lymphoma cells were obtained from ATCC (ATCC TIB160, Rockville, MD). The viable membrane dye PKH-2 and labelling diluent used to stain YAC-1 cells were purchased from Zynaxis Cell Sciences Inc. (Malvern, PA). Propidium iodide (PI), used to determine cytotoxicity was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade and obtained commercially.

Chemical exposure

Fischer 344 rats were injected I.P. with a single dose of PFDA (20 mg/kg or 50 mg/kg) in 50% propylene glycol/water 8 days or 30 days prior to sacrifice. Pair-fed rats were injected with an equal volume of 50% propylene glycol/water without PFDA.

Pair-feeding

The amount of food consumed by PFDA-treated rats was determined daily, and pair-fed rats received the same amount of food. Control rats which were fed *ad libitum* were included in the study.

Immunization

KLH (5 mg/ml) was prepared in sterile normal saline. Rats were injected with KLH (0.2 ml) in the caudal tail fold. The initial injection was at 14 days and the second injection was at 7 days prior to sacrifice.

Antibody Production

8 days or 30 days after PFDA treatment, rats were anesthetized with ketamine and xylosine and blood samples were obtained by cardiac puncture. Serum was isolated and stored at -20°C until analyzed. Serum samples were analyzed for KLH-specific IgG_{2a}, IgM, and IgA by enzyme-linked immunosorbent assay (ELISA) as described by Exon et al. (12). Briefly, ELISA plate wells were coated overnight at 4°C with KLH (2 mg/ml) or with the appropriate rabbit anti-rat immunoglobulin antibody at a 1:1000 dilution and were then blocked with 5% fetal calf serum in phosphate buffered saline containing 0.05% Tween. Serum samples or immunoglobulin standards at appropriate dilutions were added to the plates and incubated overnight at 4°C. Plates were washed and the appropriate horseradish-peroxidase conjugated rabbit anti-rat immunoglobulin antibodies were added at a dilution of 1:5000 followed by incubation at room temperature for 1 hour. After extensive washing, color was developed using OPD in 0.05M citrate phosphate buffer (pH 5.0) containing 0.03% H_2O_2. The reaction was stopped by the addition of 50 μl H_2SO_4 (10N). Plates were read in a Coulter Microplate Reader (Coulter Electronics, Hialeah, Fl) at 490/650 nm and absorbance values were converted to μg/ml (IgG_{2a}) or ng/ml (IgM, IgA) utilizing standard curves.

DTH Response

The DTH response was determined as described by Exon et al. (12). Briefly, heat-aggregated KLH was prepared at a concentration of 20 mg/ml in sterile normal saline by heating at 80°C for 1 hour. Heat-aggregated KLH (0.1 ml) was injected 24 hours prior to sacrifice into the right hind footpad of rats which were previously immunized against KLH. Normal saline was injected into the left hind footpad of these rats as an internal control. The same treatment was given to non-immunized rats as a negative control. Footpad swelling was measured with vernier calipers, and the DTH response was equal to the difference between swelling in the KLH-injected footpad and swelling in the normal saline-injected footpad.

Natural Killer Cell Assay

The NK cytotoxicity assay was performed as described by Slezak and Horan (13). Briefly, YAC-1 cells (target cells) were stained with the fluorescent, viable membrane dye PKH-2 according to manufacturer's instructions (Zynaxis, Malvern, PA). Cultures containing 100:1, 50:1, and 25:1 nonadherent spleen effector cells:target cells in a total volume of 200 μl were prepared in 96-well round bottomed microtiter plates. Cultures were centrifuged at 50 x g to promote conjugation and were incubated at 37°C for 4 hours. After the incubation period, cultures were centrifuged at 400 x g, and 100μl of the medium was removed. Propidium Iodide (PI) (25μg/ml) was added in an equivalent volume to determine viability. Cells were analyzed on an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL) using an exciting wavelength of 488 nm with a laser power of 300 mW. Filters used to select for different parameters were: forward angle light scatter (FALS), PI red fluorescence (635 nm band pass, 550 nm long pass dichroic, and 600 nm short pass dichroic filters), and PKH-2 green fluorescence (525 band pass and 550 long pass dichroic filters). For each sample, log green fluorescence versus log red fluorescence histograms were generated. Non-specific cytotoxicity was defined as the percentage of dead target cells occurring when incubated without effector cells, and was subtracted from specific cytotoxicity which was defined as the percentage of dead target cells following incubation with effector cells over the 4 hour culture period.

Statistical Analysis

All data were analyzed by ANOVA using SAS[R] software (SAS Institute). If treatment groups differed significantly from each other ($p < 0.001$), data were then analyzed by Tukey's studentized range test for differences between groups. Treatment groups were considered to be statistically different from controls when $p < 0.05$.

RESULTS

Antibody Production

KLH-specific IgG_{2a} levels at 8 days after PFDA treatment were significantly decreased by an average of 54% at a dose of 20 mg/kg; and by an average of 69% at a dose of 50 mg/kg when compared to *ad libitum*-fed controls (Fig. 1, top). KLH-specific IgG_{2a} levels at 8 days after PFDA treatment were also significantly less than pair-fed controls (59%, 20 mg/kg; 75%, 50 mg/kg; Fig. 1, top). KLH-specific IgM and IgA levels at 8 days after PFDA treatment were not significantly different from *ad libitum*-fed or pair-fed controls (data not shown).

KLH-specific IgG_{2a} levels at 30 days after PFDA treatment were not significantly different from *ad libitum*-fed or pair-fed controls, although there was a slight increase in the level of IgG_{2a} at the 50 mg/kg dose of PFDA (Fig. 1, bottom). KLH-specific IgM and IgA levels at 30 days after PFDA treatment were not significantly different from *ad libitum*-fed controls (data not shown).

DTH Response

The DTH response at 8 days after PFDA treatment was decreased by an average of 40% at a dose of 20 mg/kg (7 out of 9 rats) and by an average of 46% at a dose of 50 mg/kg (5 out of 8 rats) when compared to *ad libitum*-fed controls (Fig. 2, top). The DTH response at 8 days after PFDA treatment was also less than pair-fed controls (39%, 20 mg/kg; 41%, 50 mg/kg). These decreases were not statistically significant by Tukey's studentized range test.

The DTH response at 30 days after PFDA treatment was decreased by an average of 38% at a dose of 20 mg/kg (5 out of 8 rats), and by an average of 47% at a dose of 50 mg/kg (7 out of 8 rats) when compared to *ad libitum*-fed controls (Fig. 2, bottom). The DTH response at 30 days after PFDA treatment was also decreased when compared to pair-fed controls (13%, 20 mg/kg; 40%, 50 mg/kg). These decreases were not statistically significant by Tukey's studentized range test.
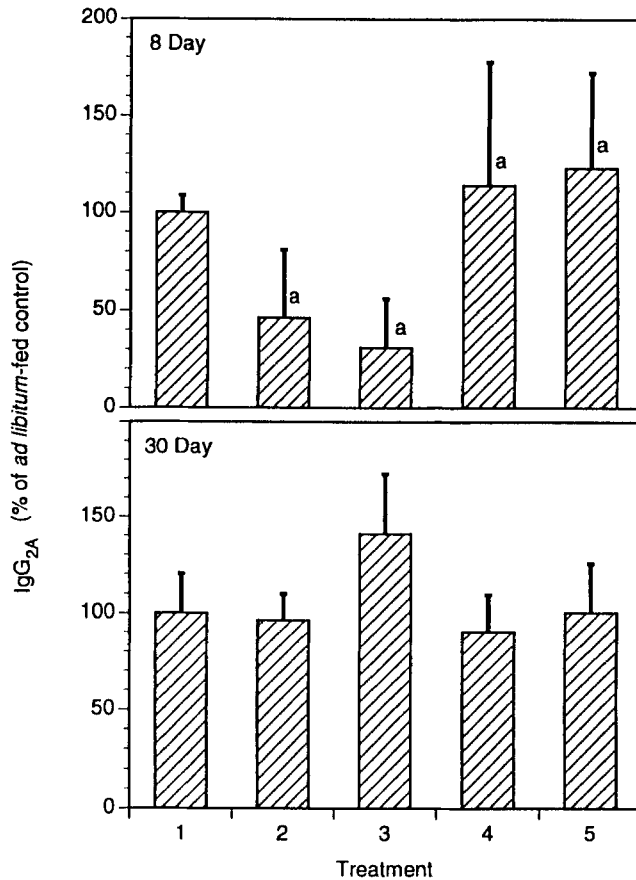
FIG. 1: IgG$_{2a}$ Production Following PFDA Treatment. Rats were injected with PFDA (20 mg/kg or 50 mg/kg) 8 days (top) or 30 days (bottom) prior to euthanasia. Serum IgG$_{2a}$ levels were determined by ELISA. IgG$_{2a}$ levels are expressed as a percentage of the mean *ad libitum*-fed control level. 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 = 20 Pair-fed control. Treatment 5 = 50 pair-fed control. (a). Significantly different from the ad libitum-fed control by Tukey's studentized range test (p < 0.05).
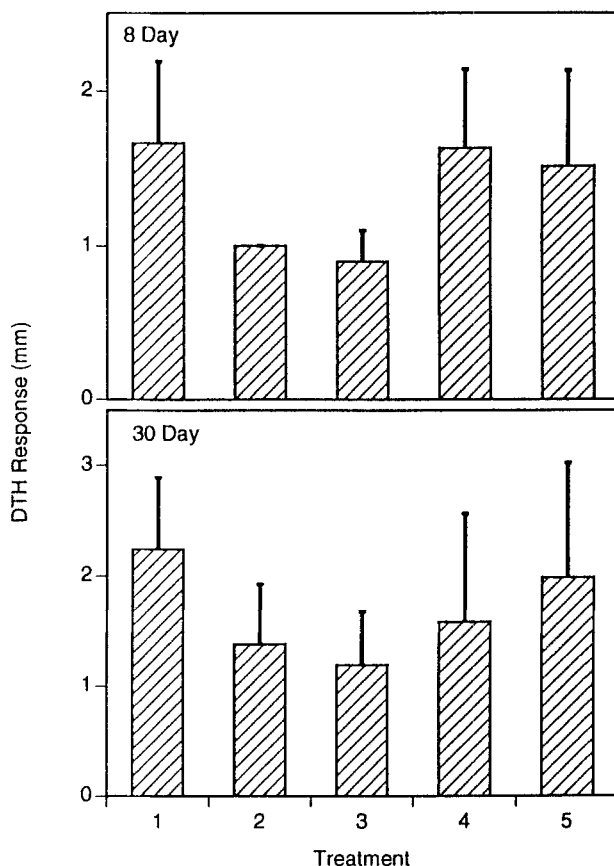
FIG. 2: <u>DTH Response Following PFDA Treatment</u>. Rats were treated with PFDA (20 mg/kg or 50 mg/kg) and were sacrificed 8 days (top) or 30 days (bottom) subsequently. Footpad thickness was measured as described in methods and materials. Mean ± s.d. is shown (n = 5). Treatment 1 = *ad libitum*-fed control. Treatment 2 = PFDA (20 mg/kg). Treatment 3 = PFDA (50 mg/kg). Treatment 4 = 20 pair-fed control. Treatment 5 = 50 pair-fed control.

<u>Natural Killer (NK) Cell Activity</u>

NK cell activity at 8 days following PFDA treatment did not differ significantly with PFDA treatment at either dose (Table 1). NK cell activity of 50 pair-fed rats was significantly greater than *ad libitum*-fed control values by 22% and 20% at the 50:1 and 25:1 Effector:Target (E:T) ratios respectively (Table 1).

TABLE 1

Natural Killer Cell Activity 8 Days After PFDA Treatment

TREATMENT	100:1 E:T RATIO	50:1 E:T RATIO	25:1 E:T RATIO
AD LIB-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 20	29.1 ± 10.0	20.3 ± 5.0	13.0 ± 5.6
PAIR-FED 50	33.1 ± 12.8	25.4 ± 11.4 (a)	17.3 ± 8.7 (a)

Rats were treated with PFDA (20 mg/kg or 50 mg/kg) for 8 days. Nonadherent spleen cells were cultured with 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 lib*-fed control by Tukey's studentized range test.

TABLE 2

Natural Killer Cell Activity 30 Days After PFDA Treatment

TREATMENT	100:1 E:T RATIO	50:1 E:T RATIO	25:1 E:T RATIO
AD LIB-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 20	34.3 ± 13.8 (a)	22.7 ± 11.5 (a)	15.8 ± 7.4 (a)
PAIR-FED 50	33.1 ± 17.1 (a)	21.6 ± 12.1 (a)	15.7 ± 8.5 (a)

Rats were treated with PFDA (20 mg/kg or 50 mg/kg) for 30 days. Nonadherent spleen cells were cultured with YAC-1 target cells at various 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 lib*-fed control by Tukey's studentized range test.

NK cell activity 30 days following PFDA treatment was significantly greater than the *ad libitum*-fed control values by 19% at the 100:1 E:T ratio (50 mg/kg) and by 16% at the 25:1 E:T ratio (50 mg/kg) (Table 2). Pair-fed NK cell activity was also significantly greater than *ad libitum*-fed control values by 17-22% at all E:T ratios (Table 2).

DISCUSSION

The effect of PFDA on humoral immunity, cellular immunity, and innate immunity was studied. The effect of PFDA on terminal KLH-specific IgG_{2a} levels was dependent on the time interval between PFDA administration and immunization with KLH. Rats which were treated with PFDA one day prior to the second immunization (8 days prior to sacrifice) had decreased terminal KLH-specific IgG_{2a} levels when compared to *ad libitum*-fed and pair-fed controls. Thus, the effect of PFDA on IgG_{2a} levels was not due to drug-induced anorexia nor was it due to propylene glycol administration. In contrast, rats which were treated with PFDA sixteen days prior to the first immunization (30 days prior to sacrifice) did not have decreased terminal KLH-specific IgG_{2a} levels when compared to *ad libitum*-fed and pair-fed controls. This lack of suppression of immunoglobulin production following the longer time interval was probably due to recovery from the initial insult and/or a decrease in the serum concentration of PFDA at the time of immunization. PFDA is eliminated from the body in the feces and it is specifically accumulated in the liver (14), thereby decreasing the amount of PFDA available to alter IgG_{2a} production.

The precise mechanism by which PFDA alters IgG_{2a} production is not known. PFDA, which is directly toxic for thymocytes in vivo (5), may also be directly toxic to B lymphocytes. An analysis of B and T cell subpopulations upon treatment with PFDA is ongoing in this laboratory. Alternatively, PFDA may disrupt cytokine production. An elevation of prostaglandin of the E series (15) or tumor necrosis factor (16) may decrease antibody production either directly or

through a decrease in interleukin-2 production. Another effect of altered cytokine production could be the inhibition of class switching. The observation that PFDA had no effect on KLH-specific IgM production 8 days after treatment, whereas it decreased IgG_{2a} production supports this theory. The effect of PFDA on cytokine production is being determined.

Although PFDA did not cause a statistically significant change in DTH response as determined by Tukey's studentized range test, PFDA did cause a trend of reduced DTH responsiveness at 8 days and 30 days following treatment in the majority of animals when compared to *ad libitum*-fed and pair-fed controls. PFDA apparently causes a longer-term effect on cellular immunity than on humoral immunity since a decrease in the DTH response is still observed 30 days after PFDA treatment. The precise mechanism by which PFDA alters the DTH response is not known. The circulating T cell population may be decreased by PFDA treatment or lymphokine production and macrophage recruitment may be reduced. PFDA may, like other perfluorocarbons (17), also be directly cytotoxic to macrophages. These data provide preliminary evidence that PFDA may cause a defect in cellular immunity, even though the observed decrease in DTH response was not statistically significant, probably because the maximum response attainable was small and the number of animals used was restricted. The DTH response is not the most sensitive method for assessing cellular immune responses, however, this method was selected because it could be used in an economical multiple immunotoxicity approach similar to that described by Exon et al. (12). The alteration in cellular immunity should be further investigated using a more sensitive method such as the mixed lymphocyte reaction.

The increase in NK activity which was observed at 30 days but not at 8 days following PFDA treatment when compared to *ad libitum*-fed controls, appears to be associated with the effect of PFDA-induced anorexia. Pair-fed rats demonstrated similar and perhaps slightly more marked increases in NK activity. It is not known if the increase in NK activity is due to an increase in the number of NK cells in the spleen or if it is due to increased activation of the normal resident NK cell population, possibly due to an increase in interferon gamma.

PFDA is a peroxisomal proliferator, and this class of compounds frequently causes hepatocarcinomas (18). PFDA has not been reported to induce hepatocarcinomas, and short-term carcinogenesis tests have been negative for PFDA (19), although PFDA has been shown to cause oxidative DNA damage in rat liver (20). The increase in NK activity reported here, if sustained for a long period of time, could prevent the establishment of hepatocarcinomas in PFDA-treated rats despite the occurrence of peroxisomal proliferation.

In conclusion, this study demonstrated dose-dependent immunomodulatory effects of PFDA. The immunomodulatory effects of PFDA may have been, in the case of NK cell activity, associated with drug-induced anorexia; whereas, the effects of PFDA on antibody production were not associated with anorexia. The precise mechanism(s) causing PFDA-induced immunomodulation have yet to be determined.

ACKNOWLEDGEMENTS

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