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Letter

Effect of perfluorooctane sulfonate (PFOS) on influenza A virus-induced mortality in female B6C3F1 mice

Keerthi S. Guruge[1], Hirokazu Hikono[2], Nobuaki Shimada[1], Kenji Murakami[3], Jun Hasegawa[1], Leo W.Y. Yeung[4], Noriko Yamanaka[1] and Nobuyoshi Yamashita[4]

[1]Safety Research Team, [2]Research Team for Advanced Biologicals, [3]Research Team for Viral Diseases, National Institute of Animal Health, Kannondai 3-1-5 Tsukuba, Ibaraki 305-0856, Japan
[4]National Institute of Advanced Industrial Science and Technology, Onogawa 16-1, Tsukuba, Ibaraki 305-8569, Japan

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ABSTRACT — Recent studies showed that perfluorooctane sulfonate (PFOS) affects the mammalian immune system at levels reportedly found in the general human population. It has been demonstrated that exposure to immunotoxic chemicals may diminish the host resistance of animals to various pathogenic challenges and enhance mortality. Therefore, the current study was carried out to characterize the effect of a 21 day pre-administration of zero, 5, or 25 μg PFOS/kg bw/day in female B6C3F1 mice on host resistance to influenza A virus infection. At the end of PFOS exposure, body/organ weights did not significantly change whereas PFOS distribution in blood plasma, spleen, thymus and lung was dose-dependently increased. PFOS exposure in mice resulted a significant increase in emaciation and mortality in response to influenza A virus. The effective plasma concentrations in female mice were at least several fold lower than reported mean blood PFOS levels from occupationally exposed humans, and fell in the upper range of blood concentrations of PFOS in the normal human population and in a wide range of wild animals. Hence, it should be important to clarify the precise mechanism(s) for excess mortality observed in the high dose group.

Key words: PFOS, Bioaccumulation, Immunotoxic, Host resistance, Mortality

INTRODUCTION

Perfluorooctane sulfonate (PFOS) is one of the newly listed Persistent Organic Pollutants (POPs) under the Stockholm Convention. Recent studies have demonstrated that exposure to perfluorinated alkyl substances can modulate rodent humoral and cellular immune functions (Dewitt *et al*., 2008; Peden-Adams *et al*., 2008). Suppression of the primary antibody response was reported in mice exposed to PFOS, at serum concentrations 14 times lower than the average concentrations of occupationally exposed workers, and in the upper range of levels reported for the general population (Peden-Adams *et al*., 2008).

Exposure to xenobiotics and the resultant alteration of immune function may effect in a modification of an organism's ability to resist infectious disease. Several studies have shown that persistent pollutants enhance susceptibility to viral, bacterial, parasitic and neoplastic challenges (Burleson *et al*., 1996). However, no studies have been done to determine the level of susceptibility to pathogens that altered due to PFOS exposure. The rodent influenza virus model has been widely used to determine the immunotoxic effects of persistent chemicals such as dioxins on viral host resistance (Burleson *et al*., 1996; Nohara *et al*., 2002). This study represents the first preliminary investigation of increase mortality caused by influenza A virus infection in mice pre-exposed to PFOS.

MATERIALS AND METHODS

Animals

Female B6C3F1 mice were obtained from Japan SLC Inc. (Shizuoka, Japan). They were acclimatized to the environment for at least 1 week before both the preliminary virus test (9- to 10-weeks old) and PFOS/virus administration (6- to 7-weeks old). Mice were housed in HEPA filtered disposable cages (Inocage; Oriental giken, Tokyo, Japan) in a light- (12 hr light-dark cycle), tempera-

Correspondence: Keerthi S. Guruge (E-mail: guruge@affrc.go.jp)

ture- (22 ± 2°C) and humidity- (50 ± 10%) controlled Bio Safety Level-2 facility at the National Institute of Animal Health (NIAH), Japan. They received food and water *ad libitum*. Bedding, food, and water were changed weekly. All procedures used in this experiment were reviewed and approved by the biosafety, animal care and ethical committees of NIAH, Japan.

Influenza virus infection

Mouse adapted influenza virus, A/PR/8/34 (H1N1), (obtained from Dr. Hideki Hasegawa of National Institute of Infectious Diseases, Japan) was used as the infectious agent. Aliquots (0.1 ml) of 1 x 10^7 plaque forming units (pfu) of virus were prepared and stored in −80°C. Virus dilution with phosphate buffer saline (PBS) was carried out immediately prior to use. Mice were anesthetized by intraperitoneal injection (i.p.) of Avertin and intranasally infected with 30 µl of virus suspension. As a preliminary test, susceptibility to the current passage of influenza A virus was examined using 9- to 10-week old female B6C3F1 mice in order to achieve a known lethal dose in control animals. Virus diluted at eight concentrations between 6.25 and 800 pfu was tested in 7 mice for each concentration. The general appearance and weight of infected mice was evaluated for twenty days. The preliminary test showed that between 100 and 200 pfu of virus inoculation caused approximately 40% mortality (data not showing). No mortality was observed using lower virus regimes tested on female mice. Therefore, the effect of PFOS on the mortality was assessed using 21 days of PFOS exposure followed by 100 pfu of virus infection.

PFOS exposure

The potassium salt of PFOS (CAS number 2795-39-3) was acquired from Fluka Chemical (Steinheim, Switzerland). Stock solution was prepared in Milli-Q water containing 0.5% Tween 20 at a concentration of 0.2 mg/ml. Dosing solutions were prepared weekly by serial dilution. Control mice received Milli-Q water containing only 0.5% Tween 20. Mice were dosed by gavage for 21 days with either vehicle, 5, or 25 µg PFOS/kg body weight/day prior to virus inoculation.

Experimental design for measuring PFOS effect on host resistance

Mice (6- to 7-weeks old) were randomly divided into 30 animals/dose group and 6 animals/cage. They were weighed weekly during the PFOS dosing period. At the end of a 21 day PFOS exposure, 3 animals from each group were randomly sacrificed by pentobarbital overdose, and blood samples were collected via cardiac puncture using heparinized syringes. Blood plasma was separated by centrifugation at 3,000 rpm for 10 min and kept at −20°C until PFOS residue analysis. Liver, kidney, lung, spleen and thymus were collected and weighed. Organs were kept at −20°C until PFOS residue analysis.

The remaining mice were anesthetized by i.p. injection of Avertin and intranasally infected with 100 pfu (in 30 µl of PBS) influenza A virus suspension. Following virus inoculation, mice were observed for health conditions and mortality twice a day and weight was measured daily for twenty days.

PFOS residue analysis

The blood plasma samples were thawed at room temperature, fortified with ^{13}C-PFOS (Wellington Laboratories, Guelph, Canada), and extracted. Analysis of PFOS in plasma (0.2 ml) was carried out using the ion pairing method (Guruge *et al*., 2005; Yamashita *et al*., 2004). The individual or pooled organ portions (approximately 0.1 g) were thawed, fortified with ^{13}C-PFOS and then 1 ml of Milli-Q water was added followed by homogenization with a Micro Homogenizing System (Tomy Seiko Inc., Tokyo, Japan) at 3,000 rpm for 3 min. The entire homogenate was used for the extraction similar to the procedure used with plasma. Matrix recoveries of ^{13}C-PFOS in plasma and organs were 93 ± 8 (mean ± S.D.) and 96 ± 8%, respectively. The limit of quantifications for plasma and organs was 0.2 ng/ml and 0.25 ng/g wet weight. The concentrations of PFOS in the samples were not corrected for recoveries.

RESULTS

At the end of the PFOS exposure, the measured organ masses were not significantly different among PFOS-treated and control groups (Table 1).The distribution of PFOS in the body increased with repeated gavage (Table 2). At the end of PFOS exposure, mean plasma concentration was significantly higher in 25 µg/kg exposure group compared to that in the 5 µg/kg exposure group. PFOS-distribution in the various samples ranked as follows: lung ≈ plasma > spleen ≈ thymus.

Fig. 1 shows the change in body weight during PFOS gavage and virus infection. There was no significant weight change due to PFOS exposure alone. The mean body weights of both PFOS-treated groups have greater decreasing tendency compared with the control group from post-infection days 4 to 11, however, statistically significant differences were observed only at day 9 (P = 0.04 for 5 µg/kg group and P = 0.02 for 25 µg/kg group, Dunnett's test).

Table 1. Mean body (g) and organ mass [a] of adult B6C3F1 female mice dosed with PFOS for 21 days [b]

Dose	Body weight	Spleen [b]	Thymus [b]	Liver [b]	Kidney [b]	Lung [b]
Control	20.4 ± 0.95	0.34 ± 0.04	0.36 ± 0.07	3.7 ± 0.24	1.1 ± 0.04	0.79 ± 0.13
5 µg/kg/bw/day	20.1 ± 0.45	0.31 ± 0.03	0.32 ± 0.05	3.7 ± 0.13	1.1 ± 0.03	0.67 ± 0.08
25 µg/kg/bw/day	20.2 ± 0.33	0.31 ± 0.02	0.26 ± 0.03	3.9 ± 0.27	1.2 ± 0.03	0.67 ± 0.03

[a] Organ mass = organ weight (g)/ body weight (g) x 100.
[b] Data are reported as mean ± S.E. (n = 3).

The survival rate at each time point of female B6C3F1 mice exposed to PFOS followed by infection with virus is shown in Fig. 2. The mean survival time based on 20 days observation period was 14.1, 13.2, and 11.4 days in the control, 5 and 25 µg PFOS/kg bw/day groups, respectively. There was no significant difference in survival time among the three groups ($P > 0.05$, Kaplan-Meier log-rank test). The survival rate of the mice on day 20 after virus infection was 46%, 30% and 17% in the control, 5 and 25 µg PFOS/kg bw/day groups, respectively. A significantly dose-dependent increase in mortality was observed with PFOS exposure ($P = 0.014$, Cochran-Armitage trend test). Additionally, the eventual survival rate differed significantly between 25 µg PFOS/kg bw/day group and control group ($P = 0.035$, logistic regression Wald test). There was no statistically significant difference between 5 µg PFOS/kg bw/day group and control group ($P = 0.28$, logistic regression Wald test).

DISCUSSION

Exposure to higher levels of PFOS often produces significant reduction in rodent body weight and this may influence their immunological outcome. Therefore, we have selected only two PFOS doses (5 and 25 µg PFOS/kg bw/day) for this experiment to try to reproduce environmentally existing blood levels in humans and wildlife. In our exposure regime, there was no significant change in body weight observed among all three mice groups at the end of PFOS exposure. Likewise, no significant change in organ mass was found for immune-responsive organs. The results for body and organ weight gain were similar in both male and female mice exposed to PFOS at comparable exposure levels (Peden-Adams *et al*., 2008). Nevertheless, the body weight reduction during virus infection was clearly greater in PFOS dosed mice compared to the controls. Therefore, PFOS accumulation is likely superimposed on viral illness in mice to increase mortality.

The mean plasma levels of PFOS at the beginning of virus infection were 189 ± 14 and 670 ± 47 ng/ml wet wt. in 5 and 25 µg PFOS/kg bw/day exposed groups, respectively (Table 1). Our data are similar to recently published serum levels in female B6C3F1 mice exposed to a comparable PFOS exposure regime (Peden-Adams *et al*., 2008). The PFOS concentrations found in mice plasma were within the range of those reported in sera for occupationally exposed workers in Decatur, Iowa, USA (range 145 - 3,490 ng/ml wet wt.), Antwerp, Belgium (mean: 1,480 ng/ml), adult donors (range 4 - 1,656 ng/ml wet wt.) in the USA and the upper level found in the normal population in China (Olsen *et al*., 1999, 2003 and 2007; Jin *et al*., 2007). Several studies reported that concentrations of PFOS in the blood of wildlife were similar to our data for mice treated with 5 and 25 µg PFOS/kg bw/day. In the late 1990's, mean PFOS concentrations found in blood of ringed seals and bottlenose dolphins were 242 and 143 ng/ml wet wt. in European waters (Kannan *et al*.,

Table 2. Mean ± S.D. concentration (wet weight) in blood plasma and organs in adult B6C3F1 female mice exposed to PFOS

Sample type	Dose	Concentration [a]
Plasma	Control	2.1 ± 0.3
	5 µg/kg bw/day	189 ± 14
	25 µg/kg bw/day	670 ± 47
Spleen [b]	Control	< 0.25
	5 µg/kg bw/day	84
	25 µg/kg bw/day	260
Thymus	Control	< 0.25
	5 µg/kg bw/day	60 ± 5.2
	25 µg/kg bw/day	260 ± 68
Lung	Control	1.2 ± 0.2
	5 µg/kg bw/day	190 ± 65
	25 µg/kg bw/day	970 ± 145

[a] Concentrations in plasma are given in ng/ml, in other organs are given in ng/g.
Three mice for each dose were analyzed.
[b] Pooled samples.

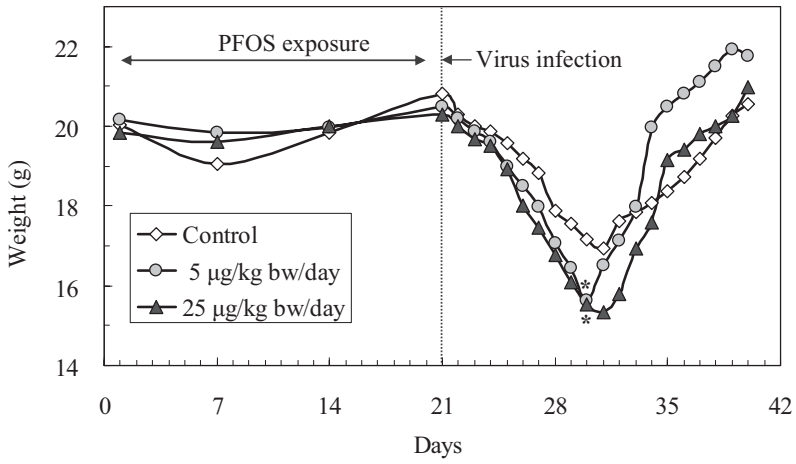

Fig. 1. Effect of PFOS on mean body weight in mice challenged with influenza A virus. Adult female B6C3F1 mice dosed with 0, 5 and 25 μg PFOS/kg bw/day for 21 days and infected intranasally. Number of animals at day 0 infection: control = 24, 5 μg PFOS/kg bw/day = 23, 25 μg PFOS/kg bw/day = 24. * Significantly different ($P < 0.05$, Dunnett's test) from the control group. Standard deviation of body weight during whole experiment was 0.30-3.8, 0.25-5.01 and 0.37-4.29 for mice dosed with 0, 5 and 25 μg PFOS/kg bw/day, respectively.

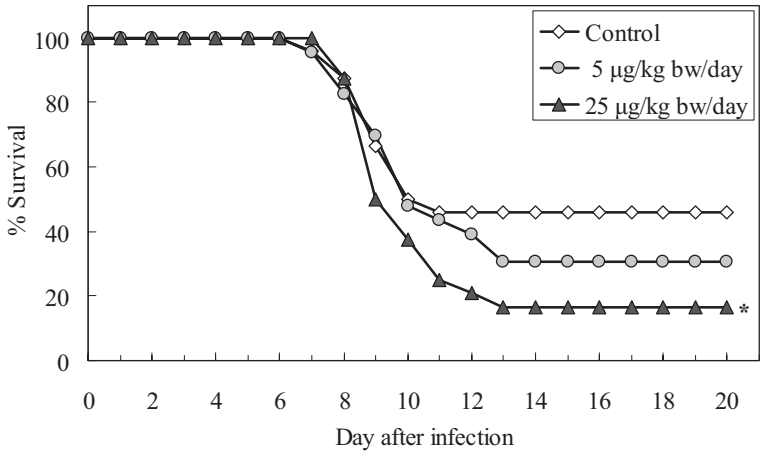

Fig. 2. Effect of PFOS on host resistance to influenza A virus. Adult female B6C3F1 mice dosed with 0, 5 and 25 μg PFOS/kg bw/day for 21 days and infected intranasally. Number of animals at day 0 infection: control = 24, 5 μg PFOS/kg bw/day = 23, 25 μg PFOS/kg bw/day = 24. * Significantly different ($P < 0.05$, logistic regression Wald test) from the control group.

2001, 2002).The PFOS concentrations in plasma of bottlenose dolphins from the Gulf of Mexico and the Atlantic Ocean were ranged from 46 to 3,073 ng/g wet wt (Houde *et al*., 2005). Interestingly, a significant association between infectious disease and elevated PFOS concentration in the livers of sea otters has been described (Kannan *et al*., 2006). As shown in Fig. 2, we observed that PFOS administration resulted in a dose-dependent enhanced mortality. In particular, 25 μg PFOS/kg bw/day exposed group exhibited a significantly reduced survival rate compared to the control group.

It is important to note that female mice treated with similar PFOS levels to this study had suppressed plasma IgM antibody production (Peden-Adams *et al*., 2008). Numerous components of the immune defense system such as cytotoxic T cells, NK cells and other humoral responses are activated and play an important role during a viral infection. Hence, any alteration of this mech-

anism may contribute to suppression of viral clearance caused by exposure to PFOS. It has been suggested that slight alterations of several immunological functions may together result in significant immunosuppression that can be detected as an increased susceptibility to infectious diseases and measured by host resistance models (Burleson *et al*., 1996). Therefore, the mechanism(s) of enhanced mortality may be related to several important immunological functions which response to viral clearance.

In conclusion, this is the first study that shows the effect of PFOS on host resistance to a pathogen in laboratory animals. Our results suggest that PFOS accumulation may associated with mortality in influenza virus-infected female mice. It will be essential to examine numerous immunological endpoints before concluding that PFOS accumulation directly leads to an alteration in host resistance to pathogens in animals.

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