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Exposure to perfluoroundecanoic acid (PFUnDA) accelerates insulitis development in a mouse model of type 1 diabetes

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ABSTRACT

Perfluoralkylated substances (PFAS) are classified as persistent, bioaccumulative and toxic substances and are widespread environmental contaminants. Humans are exposed through food, drinking water and air. We have previously reported that bisphenol A accelerates spontaneous diabetes development in non-obese diabetic (NOD) mice and observed in the present study that perfluoroundecanoic acid, PFUnDA, increased insulitis development, a prerequisite for diabetes development in NOD mice. We exposed NOD mice to PFUnDA in drinking water (3, 30 and 300 μg/l) at mating, during gestation and lactation and until 30 weeks of age. After 300 μg/l PFUnDA exposure, we report (i) increased pancreatic insulitis, (ii) increased number of apoptotic cells in pancreatic islets prior to insulitis and (iii) decreased phagocytosis in peritoneal macrophages. There was also a trend of decreased number of tissue resident macrophages in pancreatic islets prior to insulitis after exposure to 300 μg/l, and altered cytokine secretion in activated splenocytes after exposure to 3 μg/l PFUnDA. Although insulitis is a prerequisite for autoimmune diabetes, the accelerated insulitis was not associated with accelerated diabetes development. Instead, the incidence of diabetes tended to be reduced in the animals exposed to 3 and 30 μg/l PFUnDA, suggesting a non-monotonic dose response. The effects of PFUnDA exposure on increased apoptosis in pancreas and reduced macrophage function as well as accelerated insulitis development in NOD mice, may also be relevant for human insulitis. Further observational autoimmune diabetes clinical cohort studies and animal experiments for PFUnDA as well as other PFASs are therefore encouraged.

1. Introduction

Development of type 1 Diabetes Mellitus (T1DM) involves autoimmune destruction of pancreatic beta-cells, leading to insulin deficiency, with a disease development at early age in genetically predisposed individuals. There is evidence for genetic predisposition, but the increased incidence of T1DM worldwide suggests a role of environmental factors in triggering disease development (reviewed in [7,9]. Lack of specific nutrients during pregnancy (e.g. Vitamin D and long chain *n*-3 fatty acids) and viral infection during early life as well as exposure to N-nitroso compounds, air pollutants and persistent organic pollutants have been suggested to influence T1DM development in epidemiological studies [39,61,32]. There are however limited numbers of epidemiological studies investigating association between T1DM and environmental factors and causal experimental data is sparse [9].

Environmental toxins, as bafilomycin from Streptomyces infected vegetables, as well as the toxicant rodenticide Vacor, are reported to specifically decrease beta-cell function and to accelerate diabetes type 1 development in non-obese diabetic (NOD) mice, a model for T1DM [31,44], illustrating that environmental chemicals can promote T1DM development in this animal model [9].

Further, we have in earlier studies shown that exposure to bisphenol A (BPA) accelerates spontaneous diabetes development in NOD mice [6–8]. One mechanism of this accelerated diabetes development due to BPA exposure was suggested to be reduced phagocytosis by macrophages [6], leading to increased number of apoptotic cells in the pancreas and thereby accelerated insulitis development. Impaired macrophage phagocytosis was also seen after *in vitro* BPA exposure in peritoneal macrophages from both non-exposed C57/Bl6 mice and Wistar rats (Friis Berntsen et al. manuscript submitted for publication). In that study, we also observed a reduced phagocytosis in isolated mouse and rat peritoneal macrophages after *in vitro* exposure to the perfluorinated compound perfluoroundecanoic acid (PFUnDA), to a similar degree

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as observed after BPA exposure. Other perfluoroalkyl and polyfluoroalkyl substances (PFASs) like PFOS, PFNA and PFDA did not impair macrophage function to the same extent Reduced macrophage phagocytosis is one of several mechanisms in T1DM development in NOD mice [48]. Thus, we hypothesized that exposure to PFUnDA, *via* impaired macrophage function, will accelerate insulitis and T1DM in the NOD mouse model.

PFASs are substances with lipid- and water-repelling properties and are used in a wide range of consumer and industrial products including non-stick, stain-repellent, water-repellent, and fire-retardant coatings [41]. PFASs are classified as persistent, bio accumulative and toxic substances. Humans are exposed through intake of marine food and game, but also *via* air and dust from the indoor environment [30]. PFASs have been detected in human serum, breast milk and adipose tissue in more than 98% of the analysed samples. The previously most used PFASs, like perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have due to their known toxicity now widely been replaced in many consumer products with PFASs with longer carbon-chains, like PFUnDA. A longer fluorinated carbon chain has however been associated with increased toxicity [24,37,49]. The increased use of PFUnDA is accompanied by an increased PFUnDA serum level, with an increase (8%) in human sera in Northern Norway between 2001 and 2007 [46]. Typical mean human serum levels reported for PFUnDA are 0.1–0.4 ng/ml and maximal levels 0.70–1.4 ng/ml, compared to 11–23 ng/ml and 3–9 ng/ml for maximal PFOS and PFOA serum levels respectively [11,25,36,46,52,58,62].

Epidemiologic studies report immunosuppressive effects of PFAS, measured as associations between PFAS serum levels and reduced vaccine response and increased risk of infections in early childhood [26,27]. Although the evidence from epidemiologic studies regarding the association between PFAS exposure and asthma and allergy is inconsistent, the available data suggest that PFAS exposure is associated with immunotoxic effects [2,3,17,27,33,50,59,64]. Studies of immunotoxic effects of PFASs related to autoimmunity are, however, sparse, but recently, higher PFOS serum levels have been reported in T1DM patients compared to controls [54]. The aim of the present study was to investigate the effect of one selected PFAS, PFUnDA, on early stages of T1DM development in the NOD mouse model. PFUnDA was chosen since it is one of the PFASs with rising exposure levels [46], and since it was shown to impair macrophage function in our *in vitro* systems.

2. Material and methods

2.1. Mice and exposure conditions

Female (94) and male (46) NOD/ShiLtJ mice from Jackson Laboratory (Maine, USA) were used for breeding at 8 and 10 weeks of age, respectively. The female mice were randomized into 4 groups that were exposed through drinking water from the time of mating and throughout the life time of the offspring. The mating period was set to one week with vaginal plug-check twice a day and exchange of males if no vaginal plug was detected during the first two days. The males were euthanized after the mating period of one week. The 4 exposure groups included: (1) negative control (autoclaved water only), (2) PFUnDA3 μg/ml (CAS: 2058-94-8, >96% purity, Santa Cruz Biotechnology, Dallas, US), (3) PFUnDA 30 μg/ml and (4) PFUnDA 300 μg/ml. The exposure doses correspond to about 0.417, 4.17 and 41.7 μg/kg bw/day (calculated based on mean mouse weight of 23 g and mean measured volume of drinking water consumption of 3.2 ml/day at 10 weeks of age). The lowest exposure level of PFUnDA was chosen at a dose corresponding to 3 times the TDI (tolerable daily intake) for PFOS (0.15 μg/kg bw/day as set by EFSA [18]), since there is no TDI or estimated intake available for PFUnDA. Estimated total intake of PFOS and PFOA is reported to be between 0.71–2 and 13–83 ng/kg bw/day respectively in adults, while calculated levels in infants ranges between 9.4–26 and 13–83 ng/kg bw/day respectively for PFOS and PFOA [23,30]. The lowest exposure dose chosen for PFUnDA to NOD mice in this study is in the range of human environmental exposure, about five times higher than the maximal calculated intake of PFOA in human infants.

Only female offspring were selected at the time of weaning, since insulitis and diabetes development is most prevalent in female mice [35]. The perfluorinated substance PFUnDA was dissolved in deionized autoclaved water heated to 60 °C. Controls received similar water without PFUnDA. The water bottle was changed once every second week and filled up once a week. The mice had ad libitum access to feed (Harlan Teklad 2919 irradiated) and water and were exposed to a 12 h light/12 h dark cycle and 35–75% humidity. To keep the dams as the statistical unit, female siblings from each dam were separated and divided into two sub-groups, 4–5 mice per cage; (i) short term exposure for histological examination, splenocyte and peritoneal macrophage function and (ii) long time exposure for diabetes incidence surveillance. The short term exposure group was divided into groups with exposure until 7 and 11 weeks of age, used for histological examination of pancreas and collection of splenocytes and peritoneal macrophages. All experiments were performed in conformity with the laws and regulations for experiments with live animals in Norway and were approved by the local representative of the Norwegian Animal Research Authority (FOTS number 6687).

2.2. Serum glucose measurements

Serum glucose was monitored weekly (from 6 to 30 weeks of age) in female offspring in the long term exposure group in blood sample from *vena saphena* using an Accu-Check Aviva blood glucose meter (Roche Diagnostics GmbH, Mannheim, Germany). Animals with two subsequent (with 24 h interval) glucose measurements at or above 13.9 mmol/l were considered diabetic, and were euthanized.

2.3. Histological evaluation

For histological evaluation, pancreas were collected from 8 mice (at 7 and 11 weeks of age respectively), fixed in formalin, embedded in paraffin and processed as previously described before hematoxylin and eosin staining [8]. For each mouse 6 sections at different depth of the pancreas were examined and all islets present in the sections (10–15 islets/section) were graded for insulitis according to the area of an islet infiltrated by lymphocytes. 0% infiltration = grade 0, periinsulitis and up to 10% infiltration = grade 1, 10–49% infiltration = grade 2, 50–74% infiltration = grade 3 and 75–100% infiltration = grade 4, as illustrated previously in [8]. For each section, an overall grade was assigned which correlated to the highest grade detected in at least 3 islets, since all pancreas samples studied had islets with all insulitis grades represented. Then, the final grade for each pancreas/mouse was set to the highest grade determined for the 6 analysed sections. The mean insulitis grade for each exposure group corresponds to the mean of the final grade for each pancreas/mouse.

Sections of the formalin fixed pancreas were also stained overnight with antibodies towards F4/80 (tissue resident macrophages, AbD Serotec, Oxford, UK, 1:50) and active caspase-3 (apoptotic cells, Cell Signalling Technology, Beverly, MA, USA, 1:400), as previously described [8]. For each antibody staining and insulitis grade, the number of positive cells per islet was counted in two pancreatic