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Carbon black nanoparticle exposure during middle and late fetal development induces immune activation in male offspring mice

Yasser S. El-Sayed [a,b,*], Ryuhei Shimizu [c], Atsuto Onoda [c], Ken Takeda [a,c], Masakazu Umezawa [a,c]

[a] The Center for Environmental Health Science for the Next Generation, Research Institute for Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
[b] Department of Veterinary Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, Egypt
[c] Department of Hygienic Chemistry, Graduate School of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

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ABSTRACT

Increasing exposure to nanoparticles (NPs) has raised concerns regarding their health and safety profiles in humans and animals, especially in developing organisms, which may display increased sensitivity to NP toxicity. The present study examined the effects of gestational exposure to carbon black NP (CB-NP) on the development of the offspring immune system. Pregnant mice were exposed to CB-NP (95 μg/kg body weight) by intranasal instillation on gestational days 9 and 15. The thymus and spleen were collected from their offspring mice on postnatal day (PND) 1, 3 and 5. Thymocyte and splenocyte phenotypes were examined by determining the expression of cell-surface molecules using flow cytometry. Gene expression in the thymus and spleen was examined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Prenatal exposure to CB-NP increased total thymocytes and their immunophenotypes ($CD4^-CD8^-$ and $CD4^+CD8^+$ cells). It also induced an increase in total lymphocytes, and $CD4^-CD8^-$, particularly $CD3^-B220^-$cells, at PND 5 in the spleen of newborn male offspring, reflecting the stimulation of immature splenocytes. Furthermore, mRNA expression of genes related to the induction of peripheral tolerance (*i.e.* thymic Traf6) was upregulated. These data suggest that respiratory exposure to CB-NP during middle and late gestation may have allergic or inflammatory effects in male offspring, and may provide initial information on the potential developmental immunotoxicity of nanoparticles.

1. Introduction

The rapid development of nanoscience has been associated with concerns about the possible health impacts of nanoparticles (NPs). The small size of NPs means that they have a larger relative surface area per mass in comparison to bulk-size particles of the same material; this feature often makes NPs more toxic and inflammogenic (Duffin et al., 2007). Their small size also enables certain NPs to cross cell membranes and translocate from the environment into the organism (Stone et al., 2007). The lungs and airways are the most important exposure sites for involuntary exposure to NPs. Respiratory exposure to NPs elicits local pulmonary effects (*i.e.* an inflammatory response) (Brown et al., 2000; Jacobsen et al., 2009; Wilson et al., 2002), and can also translocate from the lungs into circulation and reach secondary target organs (heart, liver, brain, and testicles) (Kreyling et al., 2002; Oberdörster et al., 2002) and the developing fetus (Umezawa and Takeda, 2011). The immunotoxic potential and ability of various NPs to alter immune responses has been documented, including poorly soluble NPs of low toxicity, such as nano-sized titanium dioxide (TiO_2) and carbon black (CB) (Di Gioacchino et al., 2011; Hussain et al., 2012; Tin Tin Win et al., 2006). NP-induced oxidative damage could be one of the leading factors causing an immune imbalance because oxidative stress plays an important role in the pathogenesis of allergies and asthma (Hussain et al., 2009, 2010). Many types of NPs have been shown to produce oxidative stress under *in vivo* (Oberdörster, 2004; Park and Park, 2009; Trouiller et al., 2009) and *in vitro* (Hussain et al., 2009; Park and Park, 2009; Shvedova et al., 2003) conditions.

Abbreviations: CB, carbon black; CB-NP, carbon black nanoparticle; cDNA, complementary DNA; GD, gestational days; nm, nanometer; NPs, nanoparticles; PND, postnatal day; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Th, T helper; TiO_2, titanium dioxide.

* Corresponding author. Department of Veterinary Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, Egypt. Tel.: +20 10 01085567, fax: +2-045-3320570.
E-mail addresses: yasser_tf@yahoo.com, elsayed-ys@vetmed.dmu.edu.eg (Y.S. El-Sayed).

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Additionally, the immunotoxic effects of NPs include their ability to affect T helper cell type 1 (Th1)/Th2 balance (adaptive immune response) (van Zijverden et al., 2000) and to induce or modify the maturation and differentiation of dendritic cells (Park et al., 2010; Yoshida et al., 2010).

Based on the data collected thus far on different chemicals, drugs and pollutants, the developing immune system can be considered to be significantly more sensitive to xenobiotic insults than the adult immune system (Di Gioacchino et al., 2011). Moreover, there is increasing concern that exposure to NPs during sensitive stages of development (intrauterine life) may predispose the developing organism to diseases later in life. Indeed, experimental studies have revealed that exposure to particulate matter in ambient air is associated with adverse pregnancy outcomes (Hougaard et al., 2008), such as premature birth, reduced birth weight and small size for gestational age (Shah et al., 2011; Takeda et al., 2011), due to intrauterine growth restriction (Xu et al., 2009). It is suggested that the fetus is affected either directly by particles translocating through the placenta (Takeda et al., 2009) and by altered placental function (Yamashita et al., 2011); or indirectly by circulating cytokines or other secondary messengers that are activated in response to inflammation and/or oxidative stress in exposed mothers (Hougaard et al., 2011; Kannan et al., 2006). Maternal exposure to nano-sized TiO_2, CB or diesel exhaust particles seems to promote offspring immune responses to allergens (Fedulov et al., 2008). CB nanoparticles (CB-NP) are attractive benchmark nanoparticles because their toxic effects have been well characterized. In the present study, CB-NP was used as a model nanoparticle to investigate the hypothesis that maternal respiratory exposure to NPs during middle and late pregnancy affects development of lymphoid organs, primarily the offspring's thymus and spleen.

2. Materials and methods

2.1. Carbon-black nanoparticles

PRINTEX 90®, purchased from Degussa Ltd. (Frankfurt, Germany), was used as a CB-NP. CB PRINTEX 90 is a well-characterized carbonaceous core nanoparticle that consists of carbon with less than 1% organic and inorganic impurities (Brown et al., 2000; Jacobsen et al., 2007; Wilson et al., 2002). The primary particle size and surface area of CB-NP are 14 nm and 300 m^2/g, respectively. The particles were suspended at a concentration of 5 mg/ml in distilled water and sonicated for 30 min, followed by filtration through a 450-nm filter (S-2504, Kurabo Co., Ltd. Osaka, Japan) to remove bulk agglomeration. The peak size distribution and concentration of CB-NP in the filtrated suspension were 84.2 nm and 95 μg/ml, respectively (Onoda et al., 2014).

2.2. Animals and treatments

Pregnant ICR mice were purchased from SLC Inc. (Shizuoka, Japan). The mice were housed in a room at a controlled temperature (23 ± 1 °C) and humidity (55 ± 5%), with a 12-h dark/light cycle and *ad libitum* access to food and water. The pregnant mice were put into an anesthesia box filled with halothane and removed from the box when they began to sleep. The mice were immediately laid on their backs and treated with 1 ml/kg body weight of CB-NP suspension (95 μg/ml, for the CB-NP group, $n = 11$) or distilled water (for the control group, $n = 8$) by intranasal instillation into both nostrils. The treatment was performed on gestational days (GDs) 9 and 15, which correspond to the presence of proper embryonic thymus and spleen development (Blackburn and Manley, 2004; Dietert and Holsapple, 2007; Hollander et al., 2006). After treatment of the pregnant mice and the birth of the litters, the thymus and spleen were collected from their offspring on postnatal day (PND) 1, 3 and 5 under sodium pentobarbital anesthesia. The experimental protocol used in this study is summarized in Supplementary Fig. S1. The animal experiments were performed in accordance with the institutional and national guidelines for the care and use of laboratory animals. All efforts were made to minimize the number of mice used and their suffering.

2.3. Flow cytometry

Fluorescein isothiocyanate-labeled anti-CD3 and anti-CD4 antibodies and phycoerythrin-labeled anti-CD8 and anti-B220 antibodies were provided by Abe Laboratory (Division of Immunobiology, Research Institute for Biological Sciences, Tokyo University of Science, Japan). Single-cell suspensions of thymus and spleen in RPMI-1640 (1×10^6 cells/ml) were prepared using frosted glass slides. The suspensions were washed in FACS medium (phosphate-buffered saline containing 1% fetal bovine serum and 0.1% sodium azide) and treated with anti-FcR (2.4G2), followed by staining with fluorescently labeled antibodies. The cells were then washed, resuspended in the FACS medium and subjected to analysis. Dead cells were excluded by forward light scatter gating and propidium iodide staining. The fluorescent data of 10,000 lymphocyte events per sample were acquired on a BD FACS Canto™ II (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo 7.2.2. software (Tomy Digital Biology Co., Ltd Tokyo, Japan).

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from thymus and spleen tissues with Isogen (Nippon Gene Co., Ltd. Tokyo, Japan). Total RNA (1 μg) was used as a template to make the first strand of complementary DNA (cDNA) using M-MLV Reverse Transcriptase (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR Green Real-Time PCR Master Mix (Toyobo Co. Ltd. Osaka, Japan) and primers (Fasmac Co., Ltd. Kanagawa, Japan) for the indicated genes (Table S1). The values of target genes were normalized to the expression level of the housekeeping gene, Gapdh.

2.5. Statistical analysis

All data are expressed as the mean ± standard deviation (SD), and the levels of significance are cited. SPSS statistical package

Table 1
Number and sex ratio of offspring.

Group	Number of dams	Number of offspring/dam[a]			Total offspring	Sex ratio (%) (Male/(males + females) × 100)
		Male	Female	Total		
Control	8	5.75 ± 2.92	5.38 ± 3.66	11.00 ± 5.18	89	51.69
CB-NP	11	6.27 ± 2.57	7.09 ± 2.34	13.36 ± 3.70	147	46.94

[a] Dams were allowed to deliver their pups on gestational day 19, equal to postnatal day (PND) 0. Individual pups were recorded on PND 1, and pups were counted and their sex determined. Values are expressed as mean ± SD. Abbreviation: CB-NP, carbon black nanoparticle.