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Exposure of Pregnant Mice to an Air Pollutant Aerosol Increases Asthma Susceptibility in Offspring

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Air pollution contributes to both exacerbation and development of bronchial asthma. Studies showed that coexposure to air pollution directly promotes sensitization to inhaled allergen in neonatal mice. The aim of this study was to investigate whether prenatal exposure to air pollution could also increase susceptibility to development of asthma in early life. Pregnant female BALB/*c* mice were exposed to aerosolized leachate of residual oil fly ash (ROFA, 50 mg/ml, 30 min) at 5, 3, and 1 d before delivery. Offspring were treated once at 3 d of age with ovalbumin (OVA, 5 μg) and alum (ip), an intentionally suboptimal dose for sensitization, exposed to aerosolized OVA (1%, 10 min) at 12–14 d or 32–35 d of age, and evaluated 2 d after the final exposure. The offspring of ROFA-exposed mothers (ROFA group) revealed increasing airway hyperresponsiveness (higher enhanced pause [Penh] to methacholine challenge) and elevated substantial numbers of eosinophils in the bronchoalveolar lavage flued (BALF). Histopathology revealed prominent inflammation in the lungs of ROFA group and showed increased allergen-specific IgE and IgG1 levels. Their cultured splenocytes showed an enhanced interleukin (IL)-4/interferon (IFN)-γ cytokine, indicating Th2 skewed immunity. Data indicate that exposure of pregnant female mice to an air pollutant aerosol increased asthma susceptibility in their offspring.

The increasing incidence of asthma and other childhood allergic diseases is a major public health concern (Beasley et al., 2000; Milligan et al., 1998; Wahn et al., 1997; Peat et al., 1994). Numerous etiologies have been suggested, including the potential influence of environmental exposures, such as secondhand tobacco smoke or air pollution (Gern et al., 1999; von Mutius, 2000; Wiesch et al., 1999). The relevance of air pollution to asthma is corroborated by epidemiologic evidence of exacerbation of existing asthma on high pollution days and by pollutant-mediated increases in allergy outcomes experimentally in animals (Goldsmith & Kobzik, 1999; Eldridge & Peden, 2000; Ormstad et al., 2000). The evidence for an adjuvant effect of air pollutants in the initiation of asthma is limited (Steerenberg et al., 2003), but includes some epidemiologic associations of increased asthma incidence in high-traffic areas. Both epidemiological and experimental studies reported that diesel exhaust particles (DEP), a major component of particulate air pollution in many urban areas, stimulate sensitization to allergens (Miyabara et al., 1998, Steerenberg et al., 2003). It was also found that a surrogate for ambient particulate air pollutants, residual oil fly ash (ROFA) from an oil combustion power plant, contributes to increased asthma susceptibility in young mice after coexposure to aeroallergen (Hamada et al., 1999, 2000; Steerenberg et al., 2003).

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Epidemiological studies indicate that maternal asthma is a significant risk factor for development of asthma (Liu et al., 2003). Exposure to allergen during pregnancy may promote allergy in the neonate, since the fetus is sensitized to allergen before birth by intrauterine exposure (Jones et al., 2002). There are also other pathways for maternal influence on the fetal immune systems that may prime or sensitize the developing individual for enhanced susceptibility to asthma (Szekeres-Bartho, 2002). Indeed, studies reported allergen-independent maternal transmission of asthma susceptibility using a mouse model (Hamada et al., 2003).

This model was adapted to investigate the possible effects of air pollution exposures during pregnancy on asthma susceptibility of offspring. After exposure of normal, nonallergic pregnant mice to aerosolized ROFA leachate as an experimental air pollutant, the offspring were subjected to an intentionally suboptimal sensitization protocol with ovalbumin allergen (OVA) in aluminum hydroxide adjuvant (alum). Subsequent analyses measured the development of airway hyperresponsiveness (AHR) and allergic inflammation (AI) after aerosol exposures to OVA, and the state of Th2 versus Th1 polarization of immune responses in neonatal mice with prenatal exposure to air pollutants (Ridge et al., 1996, Shearer et al., 1997). Data indicate that air pollution exposure during pregnancy increases susceptibility of neonates to allergic asthma.

METHODS

Animals

Pregnant BALB/*c* female mice were obtained commercially from Japan SLC Co. (Shizuoka, Japan) or by in-house breeding. The mice were housed in an animal facility that was maintained at 22–24°C with a 12-h dark/light cycle. All experimentation was conducted under a protocol approved by our institutional review board.

Study Protocol

Figure 1 summarizes the experimental protocols used in this study. A single large batch of residual oil fly ash (ROFA), obtained from the precipitator unit of a local power plant, was generously provided by Dr. John Godleski (Harvard School of Public Health, Boston). ROFA was suspended (100 mg/ml in phosphate buffered saline [PBS], pH 7.4) and sonicated for 10 min. After sitting for 30 min at room temperature, the ROFA suspension was incubated at 37°C with rotation for 4 h and then centrifuged at 3000 × g for 10 min. The supernatant (leachate) was removed and diluted to 50 mg/ml in PBS. The metal and elemental composition of ROFA leachate was analyzed through inductively coupled plasma–mass spectroscopy, using a sample prepared in a manner identical to that for the initial batch of ROFA leachate except for the use of analytical-grade H_2O as diluent. The ROFA leachate was found to contain

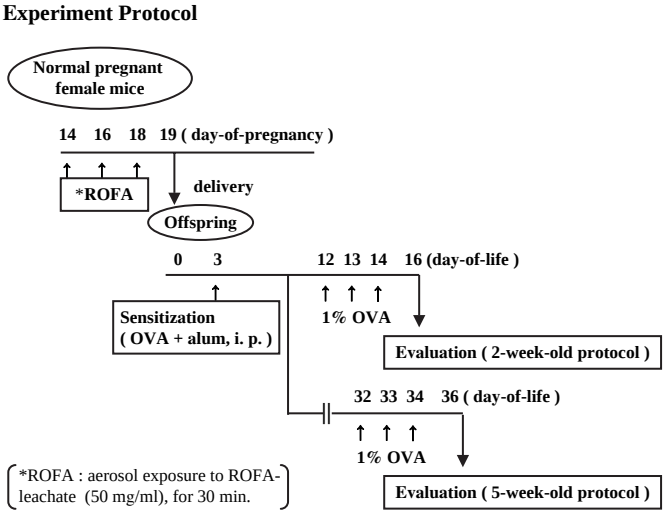

FIG. 1. Schematic summary of main protocol used in the study. Normal pregnant BALB/*c* female mice were exposed to aerosolized ROFA leachate (50 mg/ml) or PBS, 5, 3, and 1 d before delivery. The newborns received a single ip injection of OVA (5 μg) and alum (1 mg) at 3 d, followed by exposure to aerosolized OVA (1% w/v) on d 12–14 or 32–34 of life. Analyses were performed 48 h after the final allergen exposure.

the following constituents (μg/ml): Ni, 341.2; V, 323.4; Zn, 232.3; Co, 18.3; Mn, 15.8; Ca, 8.4; Cu, 6.7; Sr, 6.1; Mg, 5.0; Sb, 0.9; Cd, 0.6. No iron was detected in the supernatant produced from the ROFA sample. The pregnant mice were exposed to a nebulized ROFA leachate for 30 min/d at d 14, 16, and 18 of pregnancy (5, 3, and 1 d before delivery). As reported previously (Hamada et al. 2002), exposure to ROFA leachate induced transient neutrophils influx with maximal effect at 12 h after exposure; hence sporadic exposure was chosen in this protocol to reduce stress on pregnancy and prevent any fetal abnormalities. The aerosol was generated with a Pari-boy II nebulizer (Matsuyoshi & Co., Ltd, Tokyo) or Pari IS2 nebulizer (Sun Medical Supply, Kansas City, KS) connected to an air compressor (PulmoAID; DeVilbiss, Somerset, PA). Control mice were exposed to PBS alone. In a subset of experiments, a nickel sulfate solution in PBS with the same concentration of Ni as found in ROFA leachate (341 μg/ml) was used for aerosol exposure of pregnant mice, followed by evaluation using the same protocol as described earlier. After birth, neonatal mice were treated with a single ip injection of 0.1 ml PBS containing ovalbumin (OVA; Grade III, Sigma Chemical, St. Louis, MO, 5 μg) and alum (0.5 mg) on d 3. On d 12–14 of life, these neonates were exposed to aerosolized OVA (1%, w/v) for 10 min/d (2-wk-old mice). Some of mice were exposed to the same aerosolised OVA on d 32–34 of life (5-wk-old mice). Physiological and pathologic analy rol neonatal mice, delivered from control maternal mice, were treated in the same way. Some of the control mice were sensitized and exposed to aerosolized PBS instead of OVA (sham group).

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Adoptive Nursing Experiment

Some neonates from mothers exposed to ROFA leachate and PBS during pregnancy were switched immediately after birth in order to evaluate the role of breast milk in maternal effects. The neonatal mice in this protocol were also sensitized and exposed to OVA and evaluated as already described (same as the 2-wk-old protocol).

Pathologic Analysis

Mice were euthanized with sodium pentobarbital (Nacalai Tesque Inc. Kyoto, Japan, and Veterinary Laboratories, Lenexa, KS) and exsanguinated by cardiac puncture after opening the chest walls. Serum was prepared and stored at −20°C. The trachea was cannulated (tube size; 22 gauge [0.9 mm] for 2-wk-old and 18 gauge [1.4 mm] for 5-wk-old mice) after blood collection. Bronchoalveolar lavage (BAL) was performed three times with 0.3 (2-wk-old) or 0.8 (5-wk-old) ml of sterile PBS instilled and harvested gently. Lavage fluid (recovery volume was about 85% of instilled) was collected, centrifuged at 300 × g for 10 min, and the cell pellet was resuspended in PBS. Total cell yield was quantified with a hemocytometer. BAL differential cell counts were performed on centrifuge slides prepared by centrifugation samples at 800 rpm for 5 min (Autosmear CF-12D, Sakura Seiki, Osaka). The slides were fixed in 95% methanol and stained with Diff-Quick (International Reagent Corp., Kobe, Japan), a modified Wright–Giemsa stain, and in total 200 cells were counted for each sample by microscopy. Macrophages, lymphocytes, neutrophils, and eosinophils were enumerated. After lavage, the lungs were instilled with 15% buffered formalin. After paraffin embedding, section for microscopy were stained with hematoxylin and eosin (H&E). An index of pathologic changes in coded H&E slides was derived by scoring the inflammatory cell infiltrates around airways and vessels for greatest severity (0, normal; 1, less than one cell diameter thick; 2, 4–10 cells thick; 3, >10 cells thick) and overall extent (0, normal; 1, 25% of sample; <25–50%; 3, 50–75%; 4, >75%). The index was calculated by multiplying severity by extent, with a maximum possible score of 9. The extent of goblet cell hyperplasia in airway epithelium was scored on a similar 0–3 scale (Hamada et al., 2003).

Physiologic Analysis

Airway responsiveness of mice to increasing concentrations of aerosolized methacholine was measured using whole body plethysmography (Buxco, Wilmington, NC). Briefly, each mouse was placed in a chamber and continuous measurements of box pressure/time wave were calculated via a connected transducer and associated computer data acquisition system. The main indicator of airflow obstruction, enhanced pause (Penh), which shows reliable correlation with the airway resistance examined by standard evaluation methods, was calculated from the box waveform (Hamelmann et al., 1997). After measurement of baseline Penh, aerosolized PBS or methacholine in increasing concentrations (6, 12, 25, and 50 mg/ml) was nebulized through an inlet of the chamber for 1 min and Penh measurements were taken for 9 min after each dose. Penh value for the first 5 min after each nebulization were averaged and used to compare results across the groups.

Assay of OVA-Specific Immunoglobulin

Anti-OVA-specific immunoglobulin (Ig) E antibody was measured by enzyme-linked immunosorbent assay (ELISA) as previously described (Hamada et al., 2000, 2003). Briefly, 96-well microtiter plates (Nalge Nunc Int, Tokyo) were coated with 0.2 μg monoclonal rat anti-mouse IgE (BD Pharmingen, San Diego, CA) diluted in 0.1 *M* carbonate buffer (pH 9.5). After overnight incubation at room temperature, plates were washed with PBS–0.05% Tween and blocked with PBS–bovine serum albumin (BSA) (5% w/v, pH 7.4) for 1 h, followed by addition of serum samples. After overnight incubation at room temperature and washing, biotinylated OVA (1 μg/ml) was added to the plates. Plates were incubated for 1 h at room temperature and washed. After 1 h incubation with streptavidin–horseradish peroxidase (HRP) (Zymed, San Francisco, CA; 1/4000), the reaction was developed with TMB^{+} (tetramethylbenzidine) substrate-chromogen (Dako Japan, Kyoto, Japan). Plates were read in a microplate reader (model 3550, Bio-Rad Laboratories Inc., Tokyo) at 450 nm. For a positive control standard, a purified, monoclonal anti-OVA IgE, developed using standard hybridoma generation techniques (Palecanda et al., 1999), was used.

As with the IgE antibody, anti-OVA-specific IgG1 antibody was also measured with ELISA. Briefly, plates were coated with OVA (10 μg/ml) by overnight incubation (4°C), and washed and serum samples were incubated for 1 h at room temperature. After washing of samples with PBS–Tween 0.05%, peroxidase-conjugated goat anti-mouse IgG1 (1/2000; BD Pharmingen, San Diego, CA) was added for 1 h. After a final wash, TMB was added as substrate. As a positive control, serum pooled from OVA-sensitized and airway-challenged adult mice (in which asthma manifestation such as airway hyperresponsiveness, airway allergic inflammation and high OVA-specific IgE had already been confirmed) were used. For each assay, values from serum samples were normalized relative to the absorbance of positive control serum after subtraction of buffer-only blank values from both, and are expressed as indices (ratio of the test serum to positive control serum).

Cytokine Analysis of the Neonatal Splenic Cells and Serum of Mother Mice

Splenic cells of 2-d-old mice ($n = 6$) from mothers exposed to ROFA leachate or PBS during pregnancy were harvested, mixed and cultured with or without anti-CD3 monoclonal antibody (0.1 μg/ml; eBioscience, Kobe, Japan) for 3 d. Both

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interleukin 4 (IL-4) and interferon-gamma (IFN-γ) in culture supernatant were evaluated with commercially available ELISA kits (Endogen, Woburn, MA). The detection limits for IL-4 and IFN-γ were 5 and 10 pg/ml, respectively. Serum of the mothers in both groups were harvested 1 d after the delivery and also used to assess IL-4 and IFN-γ.

Metal Concentration of Neonatal Spleen

Spleens of 3-d-old mice (n = 6) were homogenized and combined. The concentrations of the major constituents of ROFA leachate (Ni, V, Zn, Mn) were measured by inductively coupled plasma atomic emissions (ICP) analytical methods (SRL Japan, Tokyo).

Statistical Analysis

Data are summarized as mean ± standard error. Differences among groups were evaluated using analysis of variance (ANOVA). For AHR analysis and antigen-specific IgE, differences between groups were also evaluated using nonparametric unpaired test (Mann–Whitney test) between each group. A probability value $p < .05$ was considered to be significant.

RESULTS

Susceptibility to Asthma

The negative control group in this protocol consisted of neonates from normal mother mice, exposed only to PBS aerosols during pregnancy. After single ip injection with OVA and alum (d 3 after birth) and aerosolized allergen challenge (d 12–14), these mice did not develop prominent asthma features, such as airway allergic (eosinophilic) inflammation (Figure 2), although the BAL fluid contained small numbers of inflammatory cells and airway hyperresponsiveness (Figure 3). The Penh data of this group were almost superimposed with results seen in the sham group. In contrast, offspring from mothers exposed to aerosolized ROFA leachate during pregnancy showed: (1) increased eosinophils on bronchoalveolar lavage (Figure 2); (2) airway hyperresponsiveness to methacholine (increased Penh, Figure 3); and (3) robust pathologic changes of airway allergic inflammation (eosinophil and mononuclear cell infiltration around airways and vessels, and goblet-cell hyperplasia) supported by semiquantitative scoring of histological changes (Figure 4). Similar results were seen in both protocols, after evaluation of 2- and 5-wk-old mice (see Figures 2, 3 A vs. B; Figure 4, C and D, respectively). Mice from mothers exposed to ROFA-leachate were also treated with a single ip injection of PBS alone followed by aerosol challenge with OVA. This group did not show any AHR or AI, indicating that sensitization with ip OVA was required to detect allergic responses to OVA aerosols (data not shown; see also additional negative controls for this model reported in the reference; Hamada et al., 2003). Serum levels of OVA-specific IgE and IgG1 were also significantly higher in 5-wk-old mice from ROFA-exposed mothers than in controls (Figure 5), although allergen-specific IgE was not detected in either group at 2 wk of age. Data demonstrate that mice born to ROFA-exposed mothers are more susceptible than control mice to development of an asthma phenotype in response to the OVA sensitization and challenge.

To analyze whether the maternal influence occurs during gestation (in utero) or during nursing (postnatal), the effect of adoptive nursing was examined on susceptibility to allergy. Offspring of mothers exposed to ROFA leachate or PBS during pregnancy were switched immediately after birth (e.g., 24–48 h). Neonates were then subjected to the same protocol. Control neonates nursed by ROFA-exposed mothers did not develop

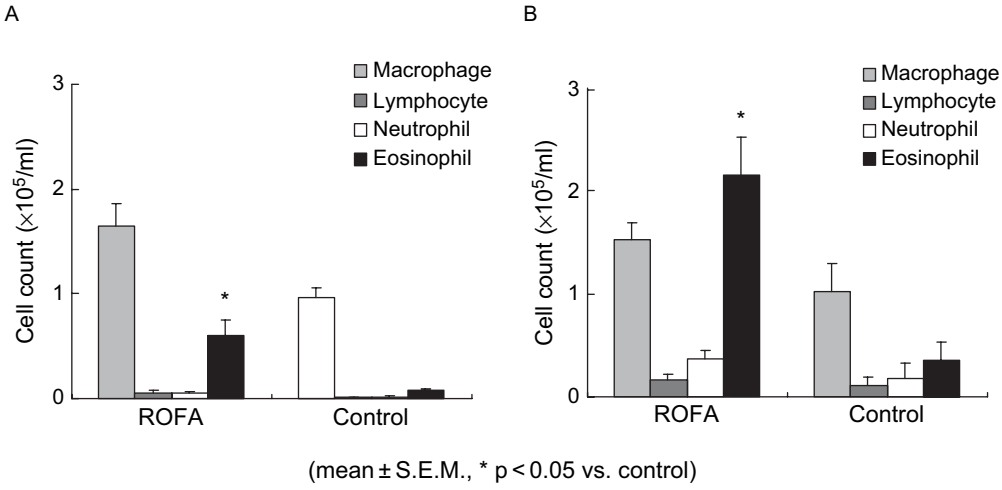

FIG. 2. Bronchoalveolar lavage analysis of (A) 2-wk-old mice and (B) 5-week-old mice. Eosinophil recruitment in both age groups is significantly higher in the offspring from mothers exposed to ROFA laechate during pregnancy (ROFA) than in the control group ($n \geq 12$ for each group, $p < .05$).

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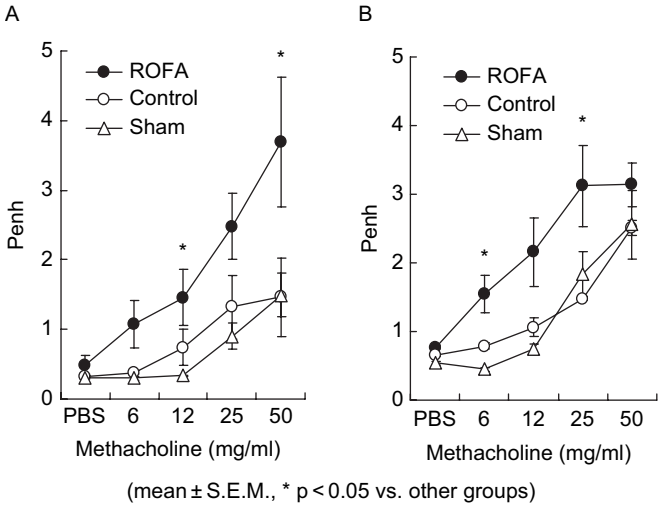

FIG. 3. Airway responsiveness to inhaled methacholine measured by whole-body plethysmography of (A) 2-wk-old and (B) 5-wk-old mice. Offspring from mothers exposed to ROFA leachate during pregnancy show increased enhanced pause (Penh) compared to offspring from control mothers, indicating airway hyperresponsiveness. Data of sham group (offspring from control mothers, sensitized to OVA and challenged to PBS instead of OVA) are superimposed to the control group ($n \geq 6$ for each group, $p < 0.05$ vs. control and sham groups).

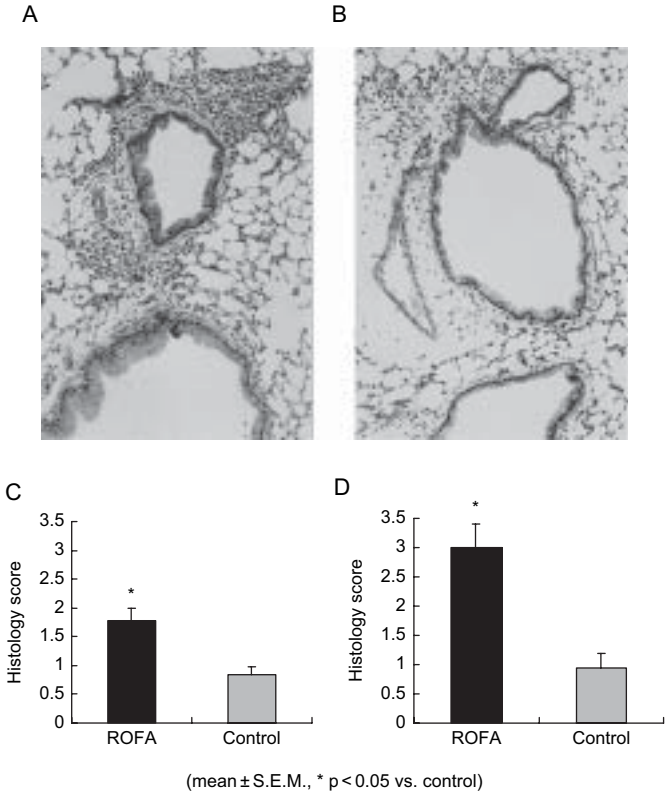

FIG. 4. Histopathologic analysis of lung tissue. Two-week-old mice from ROFA-exposed mothers (A) show increased eosinophil and mononuclear cell infiltration around airways and vessels and goblet cell hyperplasia in comparison with neonates from control mothers (B). Semi-quantitative scoring of lung histopathology in (C) 2-wk-old and (D) 5-wk-old mice showed greater allergic inflammation in the groups from ROFA-exposed mothers ($n = 5$ for each group, $p < .05$).

eosinophilic inflammation after OVA sensitization and challenge, indicating that breast milk from a ROFA-exposed mother mouse was not per se capable of increasing susceptibility (BAL eosinophil: ROFA-exposed nursed by normal mothers 0.48 ± 0.17 vs. control nursed by ROFA-exposed mothers 0.05 ± 0.02 [$\times 10^5$/ml], $n = 6$). Conversely, nursing of neonates from ROFA-exposed mothers by a control mother mouse did not prevent the development of allergy to OVA. These results suggest that breast milk is neither sufficient nor necessary for the increased susceptibility to allergy seen in offspring of ROFA-exposed pregnant mice, and indicate that the effects of ROFA occur in utero before birth.

Th2 Polarization

It was of interest to compare immune responses of ROFA-exposed or PBS-exposed mother mice and their neonates. Serum interleukin (IL)-4 and interferon (IFN)-γ concentrations in both groups of maternal mice 1 d after delivery revealed no difference (IL-4: 256.6 ± 77.7 vs. 378.9 ± 234.4 pg/ml, IFN-γ: 16.6 ± 7.4 vs. 17.8 ± 5.1 pg/ml; ROFA-exposed vs. PBS-exposed control group, respectively). Cytokine production by cultured splenocytes from 2-d-old mice, stimulated with anti-CD3 antibody, was evaluated by ELISA. IL-4 production by spleen cells from mice born to ROFA-exposed mothers was the same or quantitatively higher, and IFN-γ production was lower, than seen in cultured splenocytes from control mice. Therefore, the ratio of IL-4/IFN-γ, which is an index of Th2/Th1 balance, was higher in the mice from ROFA-exposed mothers, consistent with the greater susceptibility to develop a Th2-dominant allergic response (Figure 6) (Sehra et al., 1998; Trop & Ilan, 2002).

The aerosolized ROFA leachate (and real-world air pollutants) includes metals, as detailed in the Methods section. Analysis of metal content in the spleen tissues from offspring of ROFA-exposed mother mice shows greater concentrations of nickel (Ni; ROFA group 7.2 vs. control group 3.4 μg/g-tissue, analysis of pooled samples form 6 newborn mice) although both groups of mice showed equal levels of vanadium, zinc, or manganese (ROFA group, undetectable low, 43.7, 0.21 vs. control group, undetectable low, 44.7, 0.21 μg/g-tissue, respectively). To test the potential role of Ni in the effect of ROFA-leachate aerosols, pregnant mice were exposed to aerosols containing only Ni at the same concentration as found within the ROFA leachate (341 μg/ml). However, this exposure did not have any significant allergenic effect (BAL eosinophil: Ni-exposed group 0.11 ± 0.06 vs. PBS control group $0.08 \pm 0.02 \times 10^5$/ml, $n = 6$).

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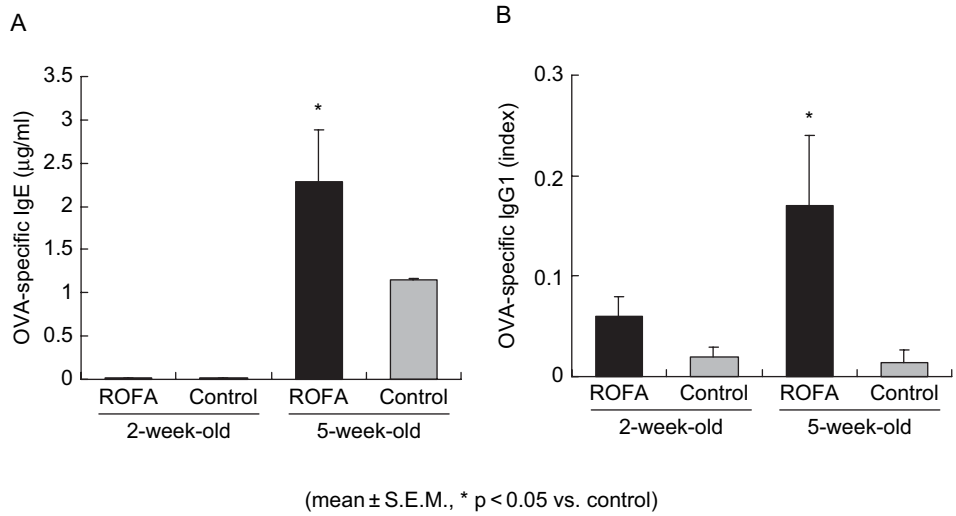

FIG. 5. Serological analysis of allergen-specific (A) IgE and (B) IgG1. Allergen-specific immunoglobulin levels in serum are significantly higher in groups from ROFA-exposed mothers than in controls ($n = 5$ for each group, $p < 0.05$).

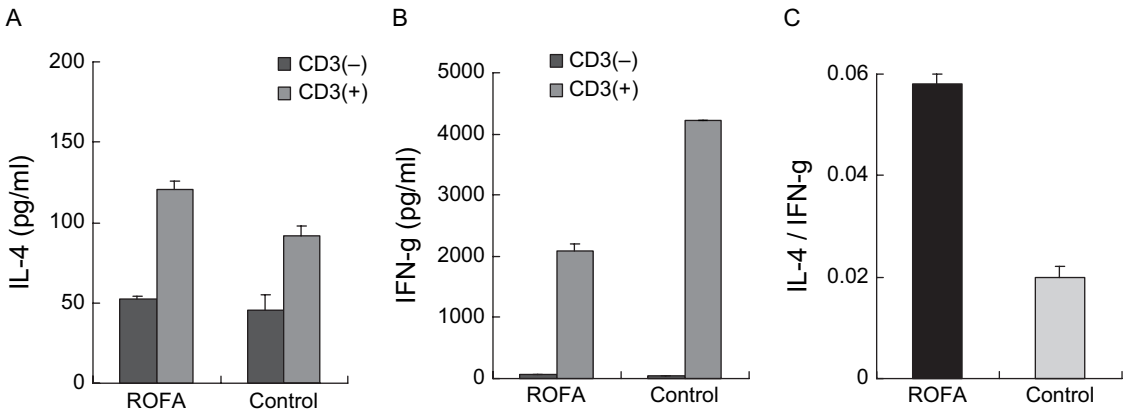

FIG. 6. Cytokine production in culture supernatants with or without anti-CD3 antibody treatment from spleen cells from 2-d-old mice. Samples from offspring of ROFA-exposed mothers showed (A) more IL-4 and (B) less IFN-γ release after CD3 stimulation, resulting in higher ratio of IL-4/IFN-γ compared to control. Data are mean ± SEM of three replicates assayed after culture of pooled splenocytes ($n = 6$ per group).

DISCUSSION

The main finding from our experiments is the observation that exposure of pregnant mice to an air pollution surrogate (aerosolized ROFA leachate) led to increased susceptibility to asthma in their offspring. Neonatal mice developed physiologic and pathologic changes characteristic of asthma following a sensitization and allergen-challenge protocol that exerted minimal effects on babies from normal control maternal mice.

Some limitations in the experimental design are worth noting. The air pollution surrogate used, ROFA leachate, is representative of only a small component of the complex mixture that comprises typical urban air pollution. Further, the concentration used for aerosol exposure of pregnant mothers is intentionally high and was chosen in past experiments based on its ability to elicit a mild-moderate neutrophil influx (Goldsmith et al., 1999). Nevertheless, as proof of principle, data support the potential for inhalation exposures during pregnancy to alter the immune system of future offspring. Additional studies using more realistic atmospheric pollutant levels with dose-response analysis are needed to better characterize the risk experimentally. Future studies will also include analysis of inert particulates or non-metal-containing mixture to establish the role of inert compounds in asthma induction.

Noninvasive plethysmography was used to detect changes in airway physiology in our mouse model of asthma. This technique reports an index related to airway hyperresponsiveness, Penh. The validity of this technique has been criticized, based on discordance with more classic, invasive measurements of lung function (Bates & Irvin, 2003 ‘Adler et al., 2004). However, the best (and arguably quite satisfactory)

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correlation between results using noninvasive plethysmography (Penh) and invasive tests of lung function is found in BALB/*c* mice developing AHR after OVA-sensitization and aerosol challenge (Hamelmann et al., 1997; Adler et al., 2004). This is precisely the system used here; together with the pathologic measures of allergic inflammation, we consider it a reasonable use of the Penh approach. This is especially true because the small size of the neonatal mice at 2 and even 5 wk of age make tracheal intubation (required for invasive measurements) essentially impossible, precluding use of those techniques.

Air pollution clearly produces exacerbation of existing asthma, but whether it contributes to the initiation of asthma is controversial (Committee of the Environmental and Occupational Health Assembly of the American Thoracic Society, 1996). The strongest arguments against a causal role for air pollution in inducing asthma derive from epidemiology. These include the observation that asthma rates increased while air pollution decreased and the striking discordance between air pollution exposures and asthma in former East and West Germany (von Mutius, 1997). However, contrary evidence, such as asthma linkage to traffic hot spots, has been reported (Nicolai, 2002). Experimentally, studies using diesel exhaust particles (DEP) or ROFA have demonstrated that these components of air pollution act as powerful adjuvants to promote allergic-type immune responses (Steerenberg et al., 2003; Takano et al., 1998; Miyabara et al., 1998; Diaz-Sanchez et al., 1997). These studies also identify potential mechanisms for these agents, since both DEP and ROFA induce or amplify Th2-dominant immune responses (van Zijverden et al., 2000; Bommel et al., 2000). As reported in Figure 6, offspring of pollutant-exposed mothers show a skewed Th2 immune response.

Our study focused on the potential of environmental exposure of pregnant mice to influence future asthma susceptibility in utero. Previous studies showed that maternal transfer of asthma risk is important in the pathogenesis of childhood asthma. The fetus may be sensitized to allergen by transplacental exposure during pregnancy (Warner et al., 2000). Epidemiological studies also show that maternal smoking during pregnancy increases asthma in children (Frischer et al., 1992; Floreani & Rennard, 1999). The present study found an antigen-independent effect of air pollution exposure, which increased susceptibility to asthma in neonates. Data suggest that pollutant exposure promotes excess or prolonged Th2-skewed immunity after birth, exaggerating the dominance of Th2 during pregnancy, which normally changes to Th1 dominance shortly after birth (Holt, 1999; Prescott et al., 1998).

It was reported that maternal transfer of metals such as lead stimulates IgE production in offspring (Snyder et al., 2000). Nickel may act as a hapten and induce allergic diseases including contact dermatitis (Cavani et al., 2003). Sudies showed elevated nickel while vanadium, zinc or manganese were not changed in offspring tissues. However, in our study, aerosol exposure using a nickel sulfate solution alone at the same concentration and pH as the ROFA leachate did not reproduce the pro-asthmatic effect. It is possible that complex interactions between metals may be required. This is suggested by our prior finding that only the combination of Ni, V, Zn Co, Mn, and Cu, but not any of these alone, may replicate the ability of ROFA leachate to produce AHR in adult mice (Hamada et al., 2002). Therefore, the mechanisms that allow exposure to particulate air pollution during pregnancy to increase susceptibility to asthma in offspring remain unknown and deserve further study.

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