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# Early life exposure to allergen and ozone results in altered development in adolescent rhesus macaque lungs

M.J. Herring [a], L.F. Putney [a], J.A. St. George [a], M.V. Avdalovic [b], E.S. Schelegle [a], L.A. Miller [a], D.M. Hyde [a,*]

[a] California National Primate Research Center, Davis, CA, USA
[b] Department of Internal Medicine, Division of Pulmonary and Critical Care, University of California, Davis, CA, USA

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ABSTRACT

In rhesus macaques, previous studies have shown that episodic exposure to allergen alone or combined with ozone inhalation during the first 6 months of life results in a condition with many of the hallmarks of asthma. This exposure regimen results in altered development of the distal airways and parenchyma (Avdalovic et al., 2012). We hypothesized that the observed alterations in the lung parenchyma would be permanent following a long-term recovery in filtered air (FA) housing. Forty-eight infant rhesus macaques (30 days old) sensitized to house dust mite (HDM) were treated with two week cycles of FA, house dust mite allergen (HDMA), ozone ($O_3$) or HDMA/ozone (HDMA + $O_3$) for five months. At the end of the five months, six animals from each group were necropsied. The other six animals in each group were allowed to recover in FA for 30 more months at which time they were necropsied. Design-based stereology was used to estimate volumes of lung components, number of alveoli, size of alveoli, distribution of alveolar volumes, interalveolar capillary density. After 30 months of recovery, monkeys exposed to HDMA, in either group, had significantly more alveoli than filtered air. These alveoli also had higher capillary densities as compared with FA controls. These results indicate that early life exposure to HDMA alone or HDMA + $O_3$ alters the development process in the lung alveoli.



## Introduction

Asthma is one of the most ubiquitous chronic disorders affecting nearly 7.1 million children in the United States under the age of 18 years ("Asthma and Children Fact Sheet." *American Lung Association*. N.p., Oct. 2012. Web. 29 July 2013). Ozone and other air pollutants may have increased adverse effects on the lungs of children compared with adults. Research indicates that early life exposure to air pollution or aeroallergens may promote the asthma phenotype and the foundation of asthma occurs in the first year of life (Miller et al., 2009; Yunginger et al., 1992; Rosenstreich et al., 1997; Mortimer et al., 2002). It is well known that air pollution affects lung function and growth in the developing lungs (Avdalovic et al., 2012; Fanucchi et al., 2006; Schelegle et al., 2003a; Schelegle et al., 2003b). Epidemiological studies in California have shown pulmonary function decrements in young adults based on early life residence in areas of high ozone levels (Tager et al., 1998). However, it is difficult to study the effects of ozone and other air pollutants in human epidemiological studies due to differences in design, methodology, and population studies (Fanucchi et al., 2006; Sousa et al., 2013).

Children with asthma sometimes outgrow their asthma in the second decade of life leading to clinical remission of symptoms, but reports vary and it appears to be roughly 50% of asthmatic children outgrow their symptoms (Bronnimann and Burrows, 1986); (Gerritsen, 2002). However, very few studies have investigated aspects of asthma remission. Asthma during development may still lead to a loss of lung function in adulthood despite symptom remission (Gold et al., 1994). In a study of adult asthma patients that were asymptomatic for at least 2 years, there was evidence of airway hyperresponsiveness after methacholine inhalation tests with or without mild airflow obstruction (Boulet et al., 1994). In addition, asthma patients in asymptomatic remission have an increased density of eosinophils and mast cells in the bronchial mucosa when compared to normal patients (van den Toorn et al., 2003). These findings suggest that asymptomatic patients may still retain underlying characteristics of the disease.

Previous studies have shown that episodic exposure to ozone exacerbates the allergic response in a monkey model of allergic airway disease (Schelegle et al., 2003a). These studies began exposure at 1 month of age with cyclic exposure periods extending to 6 months of age. At the end of the exposure period, studies showed that airway and parenchymal morphology had been remodeled (Avdalovic et al.,

*Abbreviations:* FA, filtered air; HDM, house dust mite; HDMA, house dust mite allergen; $O_3$, ozone; im, intramuscular; iv, intravenously; I, islands; B, bridges; $N_{alv}$, number of alveoli; $V_{par}$, volume of parenchyma; $V_{alv}$, volume of alveoli; $V_{ias}$, volume of interalveolar septa; $V_{ad}$, volume of alveolar duct; $V_{np}$, volume of non-parenchyma; $V_v$, volume density; $V_L$, lobe volume; $L_{v\,rb}$, length density of respiratory bronchioles; $L_{v\,tb}$, length density of terminal bronchioles; Qa, number of profiles; a/p, area per point; Pl, points hitting the lung.

* Corresponding author at: California National Primate Research Center, Hutchinson Drive and Road 98, University of California Davis, CA 95616, USA.
*E-mail address:* dmhyde@ucdavis.edu (D.M. Hyde).

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2012; Fanucchi et al., 2006). We investigated the effect of early-life 6 month episodic exposure to allergen and/or ozone on parenchymal morphology, including alveolar number and capillary density in sensitized rhesus macaques at the end of exposure. These parameters were measured in another group of macaques after 30 months of recovery from the same exposure. We hypothesized that the changes in the first 6 months of life from cyclic exposure to ozone and HDMA will continue to have structural changes indicative of altered development following exposure throughout the early years of life.

## Materials and methods

*Animals, exposure, and tissue collection.* Forty-eight male infant rhesus monkeys (*Macaca mulatta*) were selected from the outdoor breeding colony at the CNPRC, selected randomly, assigned to groups, and placed in a nursery for bottle-feeding with 24 h care at 2 days of age. Body weight between groups at this age was similar. The infants were housed in a 4.2 $m^3$ capacity exposure chambers with open temperature controlled incubators. Each chamber housed three animals. Chamber ventilation cycled at a rate of 30 changes per hr with filtered air (FA). Three animals were housed in each exposure chamber during the study. Experimental protocols were reviewed and approved by the University of California, Davis Institutional Animal Care and Use Committee. The care and housing of animals complied with the provisions of the Institute of Laboratory Animal resources and conformed to practices established by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animal studies conformed to applicable provisions of the Animal Welfare Act and other federal statutes and regulations relating to animals (Guide for the Care and Use of Laboratory Animals; National Institutes of Health, revised 1985).

The 48 rhesus monkeys were divided into two cohorts of 24 animals each. Within each cohort, there were 4 groups with six animals per group. The control group was exposed to filtered air (FA) alone. All animals were sensitized to house dust mite (*Dermatophagoides pteronyssinus*) by subcutaneous injection with alum adjuvant on the back on weeks 1, 2, and 4 as well as by intranasal instillation on weeks 1, 3, and 5. This house dust mite allergen was chosen over previously researched HDMA due to a reduction in impurities and was also used in the aerosol administration. HDMA sensitization was confirmed via skin testing with intradermal HDMA on Day 38 of the exposure protocol. The second group was exposed to house dust mite allergen (HDMA) aerosol in the exposure chambers (days 1, 3 and 5 of a 14 day cycle) for 2 h. The third group was exposed to cycles of ozone with ozone being delivered for 8 hours a day at 0.5 ppm for the first 5 days in a 14 day cycle. Ozone was generated as previously described and concentration was monitored using a Dasibi 1003-AH ozone analyzer (Dasibi Environmental Corporation, Glendale, CA) (Avdalovic et al., 2012; Schelegle et al., 2003b). This level of ozone is higher than the National Air Quality Standard of 0.075 ppm and it used to increase the result ozone has on the developing macaque lung. The fourth group was also sensitized to HDM and exposed to both HDMA/ozone. All animals in the exposure groups were exposed to 11 cyclic exposures of 5 days of exposure and 9 days of FA (Fig. 1). Exposures had a HDMA mass concentration averaging 7.05 ± 0.73 mg/$m^3$ and a mean O3 concentration of 0.500 ± 0.005 ppm (Moore et al., 2014).

The first cohort (6 month old cohort) was necropsied at the end of the 11th cycle at 6 months of age. The second cohort (36 month old cohort) remained in exposure rooms receiving FA for 30 months and the animals were necropsied at 36 months of age. Monkeys were weighed and then sedated with Telazol (8 mg/kg im) and anesthetized with Diprivan (0.1–0.2 mg/kg/min iv), with the dose adjusted as necessary by the attending veterinarian. The monkeys were euthanized with an overdose of pentobarbital sodium followed by exsanguinations through the abdominal aorta. The right middle lobe was immediately fixed with 1% glutaraldehyde—1% paraformaldehyde in cacodylate buffer at 25-cm fluid pressure (adjusted to pH 7.4, 330 mosM). Lung lobe volume measured by fluid weight displacement after fixation (Scherle, 1970). The left caudal lobe was inflated with optimal cutting temperature (OCT) diluted 1:1 in PBS and cooled to 4 °C.

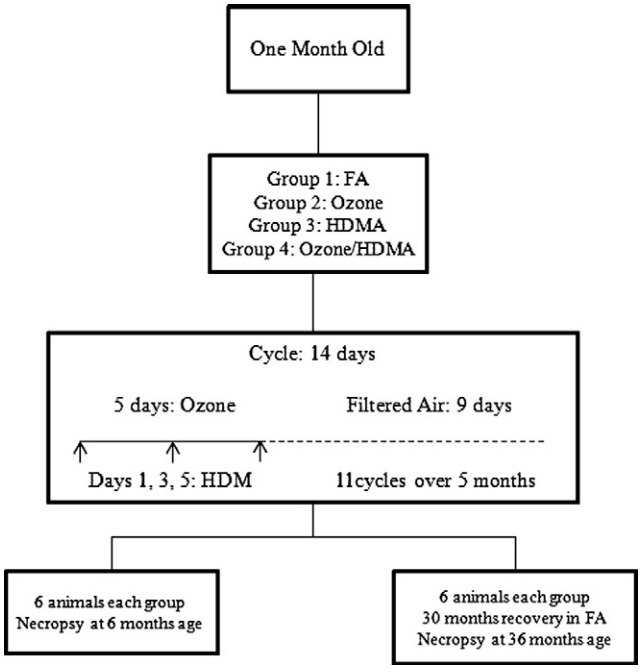


**Fig. 1.** Exposure diagram. Age at the beginning of exposure and types of exposure for each group is outlined in the diagram. Timing of HDMA and ozone exposure is shown for a typical cyclic exposure. All animals were sensitized to house dust mite by subcutaneous injection with alum adjuvant on the back on weeks 1, 2, and 4 as well as by intranasal instillation on weeks 1, 3, and 5. Sensitization to HDM was confirmed via skin testing on day 38 of the exposure protocol. Ozone exposure was 8 h/day (midnight to 8 a.m.) and HDMA exposure was 2.5 h/day on days 1, 3, and 5 in the a.m. HDM concentrations in aerosol were 435 ± 96 μg/$m^3$.

*Stereological estimates.* The right middle lobe was embedded in 4% agar–gelatin after fixation, isotropically oriented using an orientator, sliced into 5-mm slabs, and cut into 5 mm by 5 mm by 15 mm bricks. These bricks were sampled using a smooth fractionator (Gundersen, 2002; Hyde et al., 2007). The sampled tissue was embedded in paraffin, cut in 5-μm serial sections, and stained with hematoxylin and eosin. Serial sections were scanned via the Olympus VS110 whole slide scanner running software (VS-ASW-FL version 2.2 build 7667) and saved as .TIF files. Lenses used were Olympus PlanApo N2× N.A. 0.08 for the overview and Olympus PlanApo 10× N.A. 0.40 for the image. The .TIF files of the images were uploaded to a computer with VIS software version 3.4.1.5 using modules Acquisition, MicroImager, NewCast, Automated Physical Disector, and Scan Imager (Visiopharm, Denmark). All stereological sampling was done using VIS software.

The disector method was used to estimate the number of alveoli in the right middle lobe. Counting the number of entrance rings in paired sections by the disector technique allows estimation of total number of alveoli in the lung (Hyde et al., 2007; 2004; Ochs et al., 2004; Hsia et al., 2010). Two images were used from the serial sections that provided a disector height of 10 μm. Bridges and islands in the images are used for counting. Bridges are connections of interalveolar septa that appear in one section but not the other. The absence of a bridge represents an opening of the alveolus. Islands are a new isolated portion of septa. The calculations are based on the Euler characteristic of $\Delta_n = (I - B)/2$ with I representing islands and B representing bridges counted in a disector both directions, hence divided by 2. The fractionator principle was used in conjunction with the Euler characteristic to determine the total number of alveoli in the lung ($N_{alv,lung}$) where n is the number of alveolar openings per sample and SF is the total sampling