

Postnatal remodeling of the neural components of the epithelial-mesenchymal trophic unit in the proximal airways of infant rhesus monkeys exposed to ozone and allergen

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Abstract

Nerves and neuroendocrine cells located within the airway epithelium are ideally situated to sample a changing airway environment, to transmit that information to the central nervous system, and to promote trophic interactions between epithelial and mesenchymal cellular and acellular components. We tested the hypothesis that the environmental stresses of ozone (O₃) and house dust mite allergen (HDMA) in atopic infant rhesus monkeys alter the distribution of airway nerves. Midlevel bronchi and bronchioles from 6-month-old infant monkeys that inhaled filtered air (FA), house dust mite allergen HDMA, O₃, or HDMA + O₃ for 11 episodes (5 days each, 0.5 ppm O₃, 8 h/day followed by 9 days recovery) were examined using immunohistochemistry for the presence of Protein gene product 9.5 (PGP 9.5), a nonspecific neural indicator, and calcitonin gene-related peptide (CGRP). Along the axial path between the sixth and the seventh intrapulmonary airway generations, there were small significant ($P < 0.05$) decrements in the density of epithelial nerves in monkeys exposed to HDMA or O₃, while in monkeys exposed to HDMA + O₃ there was a greater significant ($P < 0.05$) reduction in epithelial innervation. In animals exposed to O₃ or HDMA + O₃ there was a significant increase in the number of PGP 9.5 positive/CGRP negative cells that were anchored to the basal lamina and emitted projections in primarily the lateral plain and often intertwined with projections and cell bodies of other similar cells. We conclude that repeated cycles of acute injury and repair associated with the episodic pattern of ozone and allergen exposure alter the normal development of neural innervation of the epithelial compartment and the appearance of a new population of undefined PGP 9.5 positive cells within the epithelium.

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Keywords: HDMA; Ozone; Allergen exposure

Introduction

The influence of aeroallergens or ozone on postnatal lung development remains an environmental health concern. We recently reported on the interaction of aeroallergen and ozone on the developing lung of rhesus monkeys (Schelegle et al., in press). We evaluated parameters of allergy, airways

resistance, reactivity, and structural remodeling in infant rhesus monkeys episodically exposed to 11 cycles of house dust mite allergen (HDMA) aerosol, ozone, or the combination of both beginning at 30 days of age and ending at 180 days of age. Rhesus monkeys were used because they have an airway branching structure and postnatal lung development similar to that of man and because of our demonstration that adult rhesus monkeys when sensitized and challenged with HDMA develop an allergic reactive phenotype similar to asthma (Schelegle et al., 2001). In monkeys sensitized with and exposed to allergen alone there was an increase in airway mucosal eosinophil content and

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proximal airway mucous cell hyperplasia without changes in lung function. In contrast in sensitized infant monkeys exposed to both allergen and ozone, eosinophils had migrated into the airway lumen, mucous cell hyperplasia had extended into the distal airways, the percentage of airways with diameters greater than 400 μm was decreased, and baseline airway resistance and airway reactivity to histamine were markedly increased. These results suggest that repeated episodes of ozone inhalation significantly amplify the allergic response and altered the postnatal development of the epithelial-mesenchymal trophic unit to produce a reactive airway phenotype (Schelegle et al., *in press*).

It has been proposed that allergen or ozone inhalation affects airway epithelial neural components (Coleridge et al., 1993; Myers et al., 2002) involved in numerous responses including reflex-mediated rapid shallow breathing, cough, bronchoconstriction, mucous secretion, and vasodilation of the bronchial circulation (Coleridge and Coleridge, 1994) and local neuropeptide-mediated bronchoconstriction, mucous secretion, increased vascular permeability, vasodilation, and epithelial cell proliferation (Barnes et al., 1991a, 1991b; Vesely et al., 1999). Allergen or ozone inhalation has been shown to result in numerous alterations in components of reflex arcs that are initiated by airway

epithelial nerves. These alterations include the function of sensory nerves (Myers et al., 2002), integration within the central nervous system (Chen et al., 2001, 2003), synaptic transmission within autonomic ganglia (Kageyama et al., 1996; Wu et al., 2001), and postganglionic neuroeffector transmission (Fryer and Wills-Karp, 1991; Ollerenshaw et al., 1989; Yost et al., 1999). While the influence of allergic airway disease on airway epithelial nerve density in adults remains controversial, with one study reporting a marked increase (Ollerenshaw et al., 1991) while another reported no change (Chanez et al., 1998), the effect of allergen or ozone inhalation on epithelial nerve density in infants whose airways are undergoing postnatal development has yet to be investigated.

In the current study, we examined small conducting airways obtained from the infant rhesus monkeys studied by Schelegle et al. (*in press*) to determine whether the postnatal development of the epithelial neural components within these airways were impacted by the repeated injury–inflammation–repair induced by the repeated episodic inhalation of HDMA, ozone, or the combination. Using immunoreactivity to the pan-neuronal marker, PGP 9.5, we observed significant decrements in the density of airway epithelial nerves in small conducting airways of infant

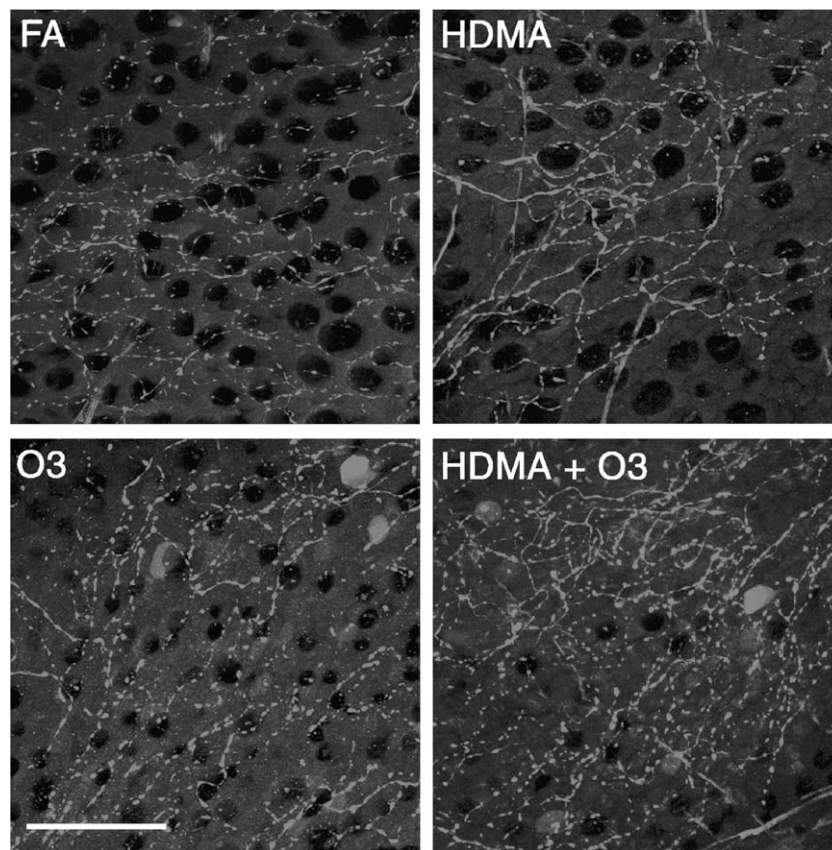


Fig. 1. Confocal images captured between the fourth and sixth airway generations of nonspecific nerves and PGP 9.5 positive cells labeled with protein gene product 9.5. Infant rhesus monkeys were exposed to filtered air (FA), house dust mite allergen (HDMA), ozone (O_3), or HDMA + O_3 . Between the fourth and sixth airway generations, there were an abundance of nerves in all treatment groups. Mag bar = 50 μm .

monkeys exposed to HDMA or ozone, while in infant monkeys exposed to HDMA plus ozone, we observed an even greater reduction in epithelial innervation. In addition, in infant monkeys exposed to ozone alone or HDMA plus ozone there was a significant increase in the number of PGP 9.5 positive cells within the airway. Using an antibody to calcitonin gene-related peptide, CGRP, as a marker for small sensory nerves, we demonstrated that CGRP-immunoreactive (IR) nerves come into close contact with the observed PGP 9.5 positive cells and that these cells did not stain for CGRP.

Methods

Animal and experimental protocol. All monkeys selected for these studies were California National Primate Research Center colony-born rhesus macaques (*Macaca mulatta*). Care and housing of animals before, during, and after treatment complied with the provisions of the Institute of Laboratory Animal Resources and conforms to practices established by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

The airway tissue examined in this study was obtained from the same 24 infant rhesus monkeys studied by Sche-

legle et al. (in press). In brief, these infant monkeys were exposed to 11 episodes of either filtered air (FA), house dust mite allergen aerosol (HDMA), ozone (O_3) or HDMA + O_3 (5 days each followed by 9 days of FA) beginning at 30 days of age. O_3 was delivered for 8 h/day at 0.5 ppm. Twelve of the monkeys (HDMA and HDMA + O_3 groups) were sensitized to house dust mite allergen (*Dermatophagoides farinae*) at age 14 and 28 days, by subcutaneous inoculation (SQ) of HDMA in alum and intramuscular injection of heat-killed *Bordetella pertussis* cells. HDMA sensitization was confirmed via skin testing with intradermal HDMA on day 38 of the exposure protocol. Sensitized monkeys were exposed to HDMA aerosol for 2 h/day on days 3–5 of either FA (HDMA, $n = 6$) or O_3 (HDMA + O_3 , $n = 6$) exposure. Nonsensitized monkeys were exposed to either FA (FA, $n = 6$) or O_3 (O_3 , $n = 6$). The methods used for ozone and allergen aerosol generation have been described previously by Schelegle et al. (in press). Airway resistance (Raw), breathing pattern (BP), arterial oxygen saturation (O_2 sat), and breath sounds were assessed, while the monkeys were anesthetized and intubated after the 6th and 10th episodes and are reported elsewhere (Schelegle et al., in press).

Following the exposure protocol, monkeys were sacrificed with an overdose of pentobarbital after being sedated

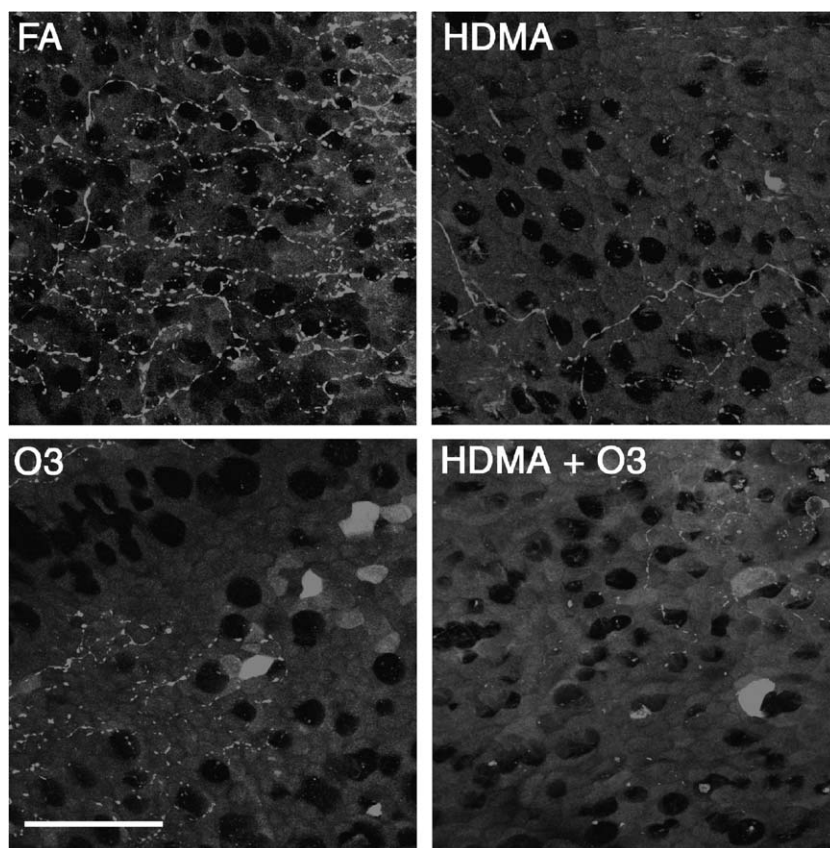


Fig. 2. Confocal images captured between the sixth and seventh airway generations of nonspecific nerves and PGP 9.5 positive cells labeled with protein gene product 9.5. Infant rhesus monkeys were exposed to filtered air (FA), house dust mite allergen (HDMA), ozone (O_3), or HDMA + O_3 . Between the sixth and seventh airway generations we see a significant decrease in the density of epithelial nerves in treated groups. Mag bar = 50 μ m.

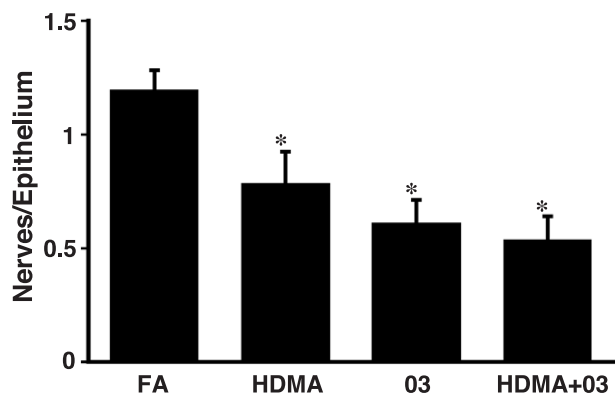


Fig. 3. Analysis from stacks of confocal sections captured between the sixth and seventh airway generations. The surface area of PGP 9.5 positive nerves per surface area of epithelium is shown. Results are mean $\pm$ SE of data obtained from five to seven airways per treatment group. There were significant differences between groups exposed to HDMA, O₃ or HDMA + O₃ and FA. ($P < 0.05$, Fisher's PLSD).

with Telazol (8 mg/kg im) and anesthetized with Diprivan (0.1–0.2 (mg/kg)/min im) with the dose adjusted as necessary by the attending veterinarian. The monkeys were then necropsied following exsanguination through the posterior

vena cava. The left cranial lobe was fixed by airway inflation (30 cm H₂O) in 1% paraformaldehyde for 4 h and the intrapulmonary airways exposed by microdissection. The mediastinal half of airways corresponding to the fourth to sixth and sixth to seventh intrapulmonary airway generations were selected for examination.

Immunohistochemistry and microscopy. Airway tissue was permeabilized with dimethyl sulfoxide (three times, 10 min each), rinsed in phosphate-buffered saline (PBS), and incubated in primary antibody (1:100 rabbit anti-PGP 9.5, Biogenesis, UK, 1:100 goat anti-CGRP, Biogenesis, UK) overnight at 4 °C, rinsed again, and incubated with a secondary antibody conjugated to goat anti-rabbit Alexa 568 (Molecular probes, Eugene, OR) or donkey anti-rabbit Rhodamine Red X and anti-goat FITC (Jackson Labs, West Grove, PA) diluted to 1:100 for 14 h at room temperature and mounted on glass slides immersed in PBS. Primary and secondary antibodies were chosen based on the demonstration of minimum cross-reactivity by the manufacture. Fluorescent images of nerves and CGRP positive mucous cells were obtained using water immersion objectives on a confocal laser scanning microscope (MRC 1024, Bio-Rad, Hercules, CA) with COMOS software

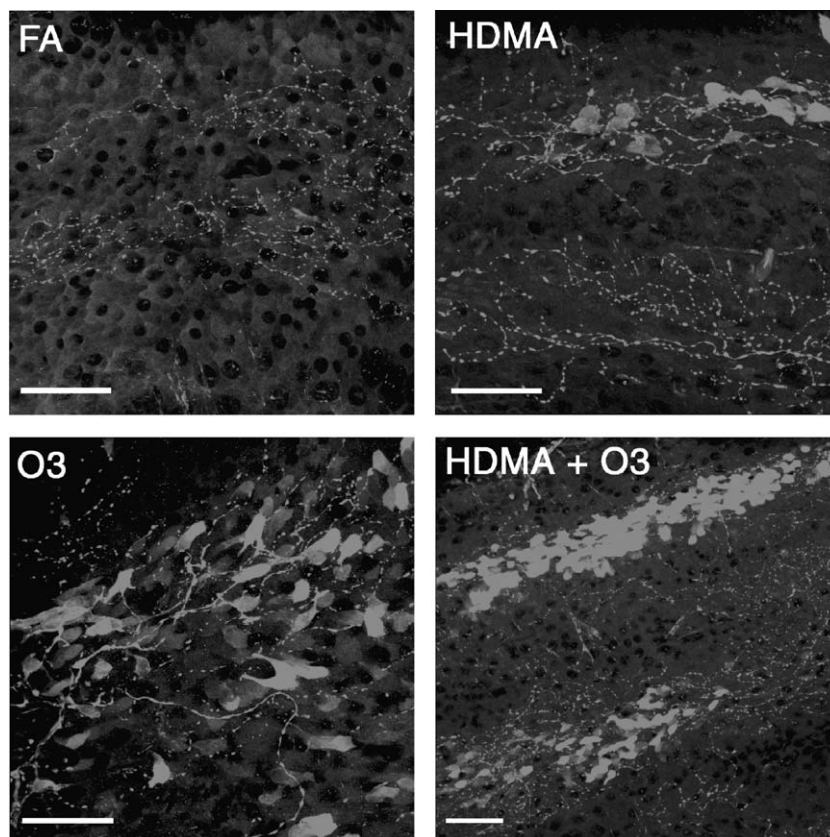


Fig. 4. Confocal images capturing airway streaks composed of nerves in monkeys exposed to filtered air (FA) and nerves and PGP 9.5 positive cells in monkeys sensitized and challenged with house dust mite allergen (HDMA) or exposed to ozone (O₃). The streaks of PGP 9.5 positive cells were most obvious in the HDMA + O₃ treatment group. Mag bar = 50 μ m.

(Version 3.2, Bio-Rad). After confocal images were captured, airways were embedded in glycol methacrylate and sectioned for further imaging.

Morphometry. In 22 of the 24 rhesus macaques studied, the number of PGP 9.5 positive cells per surface area of epithelium and the surface area of nerves per surface area of epithelium were estimated by counting through stacks of confocal images using NIH image 1.57 (written by Wayne Rasband, a public domain software available at, <http://rsb.info.nih.gov/nih-image>) and stereological techniques that were a modification of those discussed by Howard and Sandau (1992). In brief, an unbiased optical “disector” was applied to an optical series of confocal images captured using a 40× objective between the sixth and seventh airway generations. The surface area of nerves within the epithelial compartment and luminal epithelium were estimated by applying a quadratic lattice to each section and counting the number of intersections with each object of interest in both *x* and *y* directions. Because the points of the lattice are rays in the *z* direction sweeping through space, intersections in the *z* direction were recorded by transitions within and without the epithelial or nerve surface between confocal images. Objects that intersected the top section and two sides of the counting frame were excluded. Stacks of serial

sections varied from 22 to 66 images depending on the orientation of the airway surface in the dissected whole mounts.

Statistical analysis. The surface area of PGP 9.5 positive nerves per surface area of luminal epithelium was analyzed using an analysis of variance (ANOVA), where treatment groups were the grouping factor. Post Hoc analysis among the four groups were completed using Fisher’s least significant difference test (Statview, Version 5.01; SAS Institute Inc., Cary NC). All data are presented as mean plus standard error. Statistical significance was considered $P < 0.05$.

Results

Using the pan-neuronal marker PGP 9.5 and confocal imaging through the depth of the epithelial compartment of the airway, we compared the neural distribution and density of nerve fibers located within the epithelial compartment of airways from infant monkeys in four separate treatment groups; monkeys exposed to FA exclusively, monkeys repeatedly challenged with a HDMA aerosol, monkeys repeatedly exposed to O_3 , and monkeys repeatedly challenged with HDMA and exposed to O_3 .

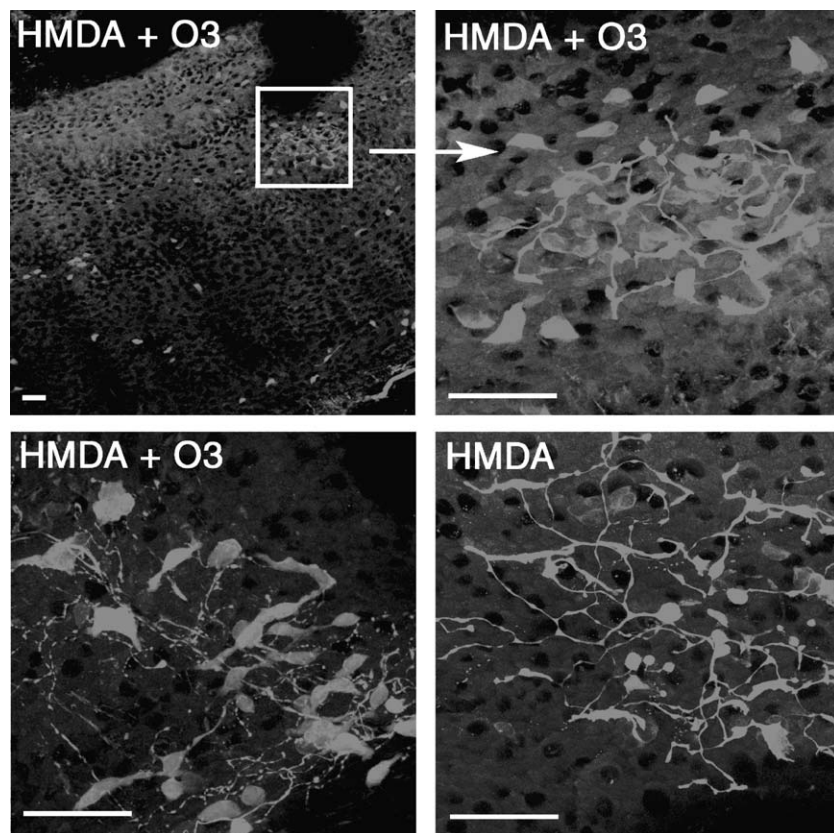


Fig. 5. Confocal images capturing clusters of airway PGP 9.5 positive cells with thick projections. Clusters were often located distal to epithelial nerve front as shown in top pair of low- and high-magnification images. Although clusters were found in house dust mite allergen (HDMA), and ozone (O_3) treatment groups, the most pronounced clusters were usually found in the HDMA + O_3 group. Mag bar = 50 μ m.

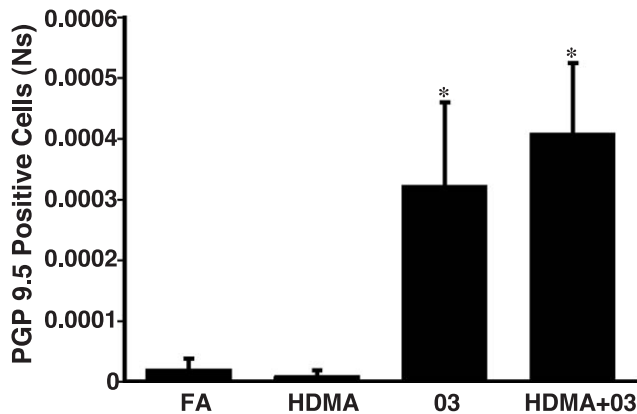


Fig. 6. Analysis from stacks of confocal sections captured between the sixth and seventh airway generations. The number of PGP 9.5 positive neuroendocrine cells per surface area of epithelium (mm²) is shown. Results are mean $\pm$ SE of data obtained from five to seven airways per treatment group. The abundance of PGP 9.5 positive cells in animals exposed to O₃ was significantly greater than animals not exposed to O₃ ($P < 0.05$, Fisher's PLSD).

Filtered air

The lungs from infant monkeys raised in FA show a regular dense network of fine nerve fibers with small varicosities throughout the epithelial compartment, both between the fourth and sixth (Fig. 1) airway generations and between the sixth and seventh airway generations (Fig. 2). In general, the nerve fibers located within the epithelial compartment of control airways between the fourth and seventh generations are evenly distributed among mucous cells, which are easily defined by the absence of a PGP 9.5 label in these cells (Figs. 1, 2, 4, and 5) (1989; Yost et al., 1999) and other epithelial populations (Figs. 1 and 2). In addition, nerve fibers found in airways of control monkeys were equally likely to be observed abutting the basal lamina or the luminal surface of the epithelium, or any space

intermediate to the two (not shown). An occasional solitary PGP 9.5 positive epithelial cell was identified within the airway, consistent with the incidence of neuroendocrine cells within monkey airways.

HDMA sensitized and challenged

The airways of monkeys sensitized to and repeatedly challenged with HDMA displayed a slight thickening of nerve fibers in proximal airways (Fig. 1), and a significant mean decrease of 34% in nerve fiber density between the sixth and seventh airway generations as compared to the control group (Figs. 2 and 3). Nerve fiber distribution within the epithelial compartment of these HDMA-challenged animals resembled that of controls. A highly variable relationship between nerve fibers and mucous cells was observed. We observed focal clusters of PGP 9.5 positive cells which often appeared to be connected to each other like beads on a string (Figs. 4 and 5), though the number of PGP 9.5 positive cells in this group of animals did not increase compared to control animals (Fig. 6). The nerves associated with these clusters contained a sensory component, as demonstrated by being immunoreactive for CGRP (Fig. 7), a known label for small sensory fibers, and favored a more apical presentation, while the PGP 9.5 positive cells were not immunoreactive for CGRP (Fig. 7).

Episodic ozone exposure

The airways of monkeys episodically exposed to O₃ displayed thicker nerve fibers in proximal airways compared to monkeys raised in FA (Fig. 1) and a significant 49% mean reduction in nerve fiber density between the sixth and seventh airway generations as compared to the control group (Figs. 2 and 3).

The density of nerve fibers of more distal airway generations was lower in these monkeys, although clusters of

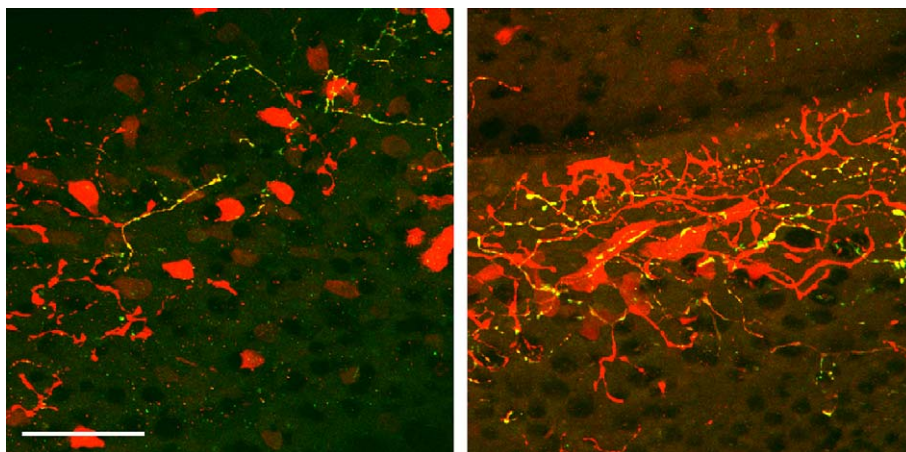


Fig. 7. Projected confocal images of nonspecific PGP 9.5 positive nerves and cells (red), and CGRP positive nerves (green) from monkeys exposed to HDMA (right panel) or HDMA + O₃ (left panel). While CGRP positive nerves were many times present in PGP 9.5 positive cell clusters, CGRP signal was not identified in PGP 9.5 positive cell projections. Mag bar = 50 μ m.

nerves, or nerves with PGP 9.5 positive epithelial cell clusters were often evident several generations distal to the generation at which the primary nerve front disappeared. The number of PGP 9.5 positive cells per surface area of epithelium between the sixth and seventh airway generations dramatically and significantly increased in this O₃-exposed group (Figs. 4 and 6). PGP 9.5 positive clusters were frequent and numerous throughout the airway. We observed little overlap between mucous cells and thick neural networks or mucous cells and PGP 9.5 clusters.

HDMA sensitized and challenged plus episodic ozone exposure

The airways of infant monkeys cyclically challenged with HDMA and episodically exposed to O₃ displayed thick and abundant nerve fibers in the proximal airway generations (Fig. 1), but a significant 55% mean reduction of epithelial innervation between the sixth and seventh airway generations (Figs. 2 and 3). Again, as in the O₃ group, epithelial innervation progressively lessened, as we examined more distal airways in these animals. In addition to the changing nerve density, we observed a more polar distribution of nerve fibers within the epithelium with most of the nerve fibers being located apical to epithelial cells. This orientation within the airway was confirmed using thin sections of tissue imbedded in glycol methacrylate (Figs. 8B and D). This orientation of nerve fibers within the epithelium was associated with a reduction in number of airway generations with extensive epithelial nerve plexuses and the appearance of clusters of PGP 9.5 positive cells within the epithelium. Clusters of PGP 9.5 positive epithelial cells along with patches of nerves were viewed several generations distal to where airway nerve density dropped off (Fig. 5, top images). The number of PGP 9.5 positive cells per surface area of epithelium was significantly higher between the sixth and seventh airway generations as compared to controls (Fig. 6) due to the abundance of PGP 9.5 positive clusters (Fig. 4) that were seen to form streaks of PGP 9.5 positive cells (Fig. 5). These streaks of PGP 9.5 positive cells extended up to 300 μ m long, were parallel to the direction of lung maturation, and contained few mucous cells. In these same animals, however, streaks of mucous cells ran parallel to and intermittent to streaks of PGP 9.5 positive cells (Fig. 4). Approximately two thirds of these PGP 9.5 positive clusters were identified at bifurcation points (Fig. 5, top left). These PGP 9.5 positive cells resembled neuroendocrine cells in appearance but not in terms of their position within the airway. Neuroendocrine cells are normally anchored at the basement membrane and extended toward the luminal surface of the airway. These PGP 9.5 positive cells, also anchored on the basement membrane, emitted projections that often followed a basal lateral direction (Figs. 8A and C). As in the HDMA-sensitized and -challenged monkeys, a subpopulation of nerves surrounding PGP 9.5 positive cells were positive

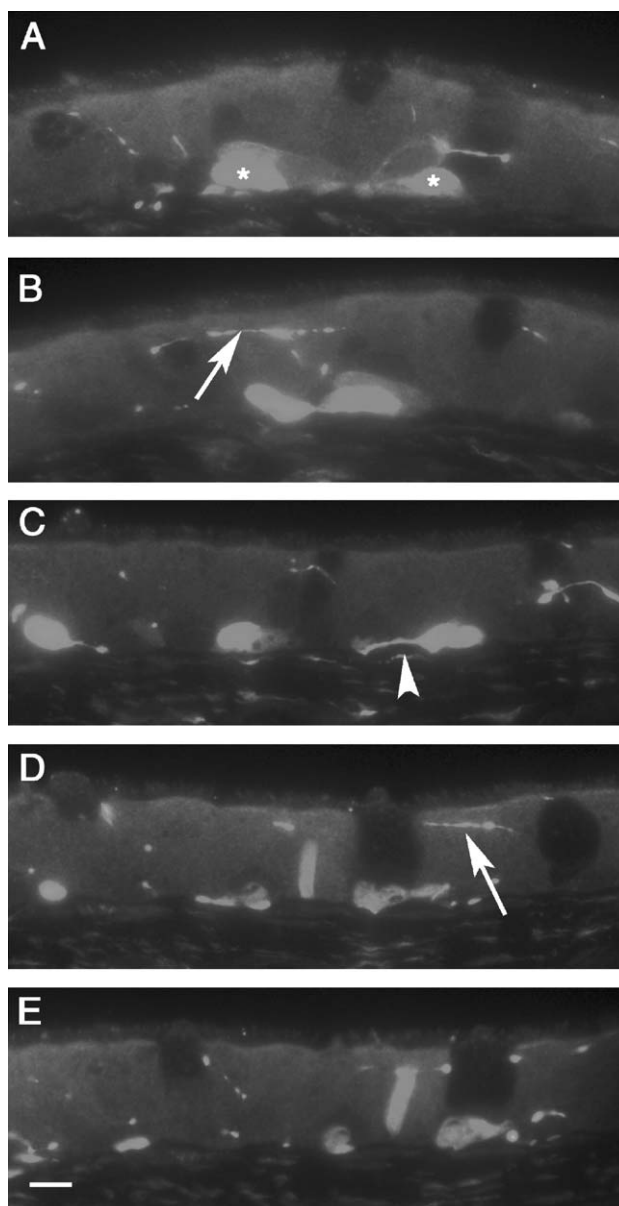


Fig. 8. Step serial sections (A–E) through a cluster of PGP 9.5 positive nerves and cells (*) from a monkey exposed to house dust mite allergen and ozone. These images display nerves (arrow) traveling at the surface of the epithelium and PGP 9.5 positive cells anchored at the basement membrane with laterally directed projections (arrowhead). Mag bar = 10 μ m.

for CGRP, indicating the presence a small afferent component within the cluster/streak (Fig. 7).

Discussion

This study demonstrates that repeated cyclic inhalation of allergen or oxidant air pollutants and especially the combination of both induces significant changes in the neural components found within the epithelial compartment of pulmonary airways of infant monkeys. In infant monkeys cyclically exposed to HDMA or O₃ between 30 and 180 days

of age, we observed two major effects: (1) a reduction in the number of airway generations with abundant nerve plexuses within the epithelium that was reflected in a significant reduction in the mean nerve density within the epithelial compartment in midlevel airways, and (2) the appearance of abnormal streaks and clusters of PGP 9.5 positive/CGRP negative cells. In addition, our data in monkeys that inhaled filtered air only demonstrate that at 180 days of age, epithelial innervation extends from the proximal to distal airways and resembles that of adult rats (Larson et al., 2003) and humans (Lamb and Sparrow, 2002).

The loss of epithelial nerve innervation between the sixth and seventh intrapulmonary airway generations in the HDMA, O₃, or HDMA + O₃ groups in conjunction with the increased diameter of nerve bundles in the nerve plexus of the airway generations just proximal indicate a neural regression or perhaps stunted nerve development. In the developing mammalian lung, nerve fiber development within the airways follows a proximal to distal progression, much like the development of the airways themselves (Sorokin et al., 1993; Tollet et al., 2002). Retarded development of epithelial nerves would be consistent with the disrupted expression and distribution of growth factors within the developing epithelial-mesenchymal zone of the airways reported by Evans et al. (2002, 2003) in tracheal tissue obtained from the monkeys studied in the current study. Such an abnormal expression of growth factors would be the result of the repeated injury and repair cycles associated with O₃ or HDMA exposure and would be consistent with the alterations in airway basement membrane (Evans et al., 2003) and structural (Schelegle et al., in press) development previously reported in these infant rhesus monkeys. However, given the lack of information on airway epithelial nerve density at 30 days of age, when the exposure regimen began, in rhesus monkeys, we cannot state with certainty that airway nerve development is retarded from this time onward in the HDMA- or ozone-exposed groups.

Whether the observed reduction in airway epithelial innervation in the exposed groups is the result of nerve degeneration or retarded development, it is another demonstration of the profound effect that allergen or ozone inhalation can have on airway neural components. Relevant to our observations on airway epithelial innervation, Myers et al. (2002) observed that allergen-induced inflammation of guinea pig airways stimulates neuronal synthesis of sensory neuropeptides in sensory ganglia. Further, these investigators provided electrophysiological and anatomical evidence that allergic inflammation causes a change in the type of airway afferent fibers that synthesize substance P, such that non-nociceptive low-threshold mechanoreceptors as well as classical nociceptive airway afferents contribute to the neuropeptide innervation of inflamed airways (Myers et al., 2002). The physiological consequence of this phenotypic change is that during airway inflammation sensory neurotransmitters may be released in the periphery and CNS not

only following nociceptive stimulation but also as a result of stimulation of low-threshold mechanosensors. In adult rhesus monkeys, Chen et al. (2001) demonstrated that sensitization and challenge with HDMA induced neuroplasticity of neurons in the nucleus tractus solitarius (NTS). These NTS neurons had an increase in baseline activity and an increased responsiveness to electrical stimulation delivered by current injection (Chen et al., 2001). Further, Chen et al. (2003) have reported that in the infant monkeys that inhaled ozone in the current study, there was a similar neuroplasticity of NTS neurons and that this neuroplasticity was mediated by substance P. Interestingly, when the same NTS neurons were studied using vagal tract stimulation they were less responsive (Chen et al., 2003). Indeed, it is tempting to link the observed reduction in epithelial innervation in the current study to the reduced responsiveness of NTS neurons to vagal tract stimulation demonstrated by Chen et al. (2003). Such a conclusion however would require neural tracing studies to demonstrate the loss of nerves with their receptor fields in the airway epithelium in the vagus nerve and the subsequent loss of their projections to the NTS.

Animals episodically exposed to ozone or ozone in combination with HDMA had an increase in the density of PGP 9.5 positive cells. These cells were similar in size to airway neuroendocrine cells but often maintained a basal lateral orientation and possessed lateral projections that are uncommon in neuroendocrine cells. Moreover, these PGP 9.5 positive cells were often loosely clustered together, with some clusters forming long (300 μ m) streaks. Monkeys exposed to HDMA alone also displayed clusters of aberrant PGP 9.5 positive cells, though their frequency was not significantly different from PGP 9.5 positive neuroendocrine cells observed in control animals. Protein gene product 9.5 expression in this unknown epithelial cell population might be linked to the chronic injury–inflammatory–repair response of the airway induced by repeated ozone or allergen exposure. The PGP 9.5 antibody binds to a molecule that is known to be a ubiquitin C-terminal hydrolase that cleaves ubiquitin from proteins and peptides that would otherwise be degraded by the proteasomes within the cell and allows recycling of free ubiquitin (Wilkinson, 2000). Ubiquitin-mediated protein degradation plays a critical role in cellular functions such as cell cycle control, DNA repair, and stress responses (Finley and Chau, 1991). Since several tyrosine kinase receptor–ligand complexes known to be important in wound healing undergo ubiquitin mediated degradation, PGP 9.5 deubiquitination could facilitate tissue repair (Mori et al., 1995). In human cutaneous tissue, PGP 9.5 staining and PGP 9.5 mRNA expression was observed in the stellate cells in the granulation tissue from chronic decubitus ulcers. Stellate cells did not label for PGP 9.5 before injury, or during the first 3 days following injury (Olerud et al., 1998), indicating that PGP 9.5 expression may be more closely linked to repair phases of wound healing. The emergence of an additional population of PGP 9.5 positive cells within the epithelium of infant animals repeatedly exposed to allergen

and ozone is not surprising given the significant impact of this protocol on both inflammatory mechanisms and structural development within the airway that were discussed previously (Schelegle et al., in press). If PGP 9.5 expression is indicative of a generalized injury–repair process, then areas of the airway that are most susceptible to injury should contain a higher concentration of these PGP 9.5 positive cells. While this unknown population of PGP 9.5 positive cells occasionally present as single cells, they were most often observed as streaks or clusters in animals exposed to O₃ or HDMA. Approximately two-thirds of the clustered PGP 9.5 positive cells were observed near the airway bifurcation points. The absence of mucous cells within the streaks of PGP 9.5 positive cells with the evident presence of mucous cells between PGP 9.5 positive streaks indicates a thinner epithelial layer within regions of the airway where streak development is evident. Both of these observations are consistent with these regions being more susceptible to inhaled irritants such as ozone.

Though the increased expression of PGP 9.5 within the cells of the airway epithelium might be attributed to an injury–repair process induced by oxidant or oxidant and allergen exposure, the transition in biochemical properties of this epithelial cell type may induce a more permanent change in physical characteristics of the cell or a trans-differentiation. Lateral projections emanating from the PGP 9.5 positive cells might indicate an attempt by the cells to connect with a larger neural/neuroendocrine system. The transdifferentiation to neuroendocrine-like cells might also enable the cells to produce peptide products like gastrin-releasing peptide that induce lung maturation.

Results from the current study demonstrate additional adverse effects of the episodic inhalation of allergen and ozone on the airway of infant rhesus monkeys. The reduction in the number of airway generations with abundant nerve plexuses within the epithelium would be expected to alter the neural signals arising from the airways, while the functional significance of the appearance of abnormal streaks and clusters of PGP 9.5 positive/CGRP negative cells in response to cyclic O₃ or HDMA exposure remains undetermined. Overall, the results of the present study have left us with more questions than answers. Do these changes in mucosal neural organization represent a disruption of postnatal development? What functional role does a decrease in neural innervation of the epithelial compartment of mid-level airways have on the increase in airway resistance and responsiveness (Schelegle et al., in press) and NTS neuron plasticity (Chen et al., 2003) reported in these monkeys? Is the new population of PGP 9.5 positive cells observed a population of epithelial cells in transition or does the appearance of PGP 9.5 positive cells represent the end product of a process of transdifferentiation? If a more permanent transdifferentiation becomes evident, then what is the progenitor cell for this new population of PGP 9.5 positive cells and what is their function within the airway? Are these changes permanent, or can they be reversed by

cessation of exposure? All of these questions would be worthy of future study.

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