Antigen-induced airway inflammation in the Brown Norway rat results in airway smooth muscle hyperplasia

K. F. Xu, R. Vlahos, A. Messina, T. L. Bamford, J. F. Bertram, and A. G. Stewart. Antigen-induced airway inflammation in the Brown Norway rat results in airway smooth muscle hyperplasia. J Appl Physiol 93: 1833–1840, 2002; 10.1152/japplphysiol.00738.2001.—Asthma is characterized by chronic airways inflammation, airway wall remodeling, and airway hyperresponsiveness (AHR). An increase in airway smooth muscle has been proposed to explain a major part of AHR in asthma. We have used unbiased stereological methods to determine whether airway smooth muscle hyperplasia and AHR occurred in sensitized, antigen-challenged Brown Norway (BN) rats. Ovalbumin (OA)-sensitized BN rats chronically exposed to OA aerosol displayed airway inflammation and a modest level of AHR to intravenously administered ACh 24 h after the last antigen challenge. However, these animals did not show an increase in smooth muscle cell (SMC) number in the left main bronchus, suggesting that short-lived inflammatory mechanisms caused the acute AHR. In contrast, 7 days after the last aerosol challenge, there was a modest increase in SMC number, but no AHR to ACh. Addition of FCS to the chronic OA challenge protocol had no effect on the degree of inflammation but resulted in a marked increase in both SMC number and a persistent (7-day) AHR. These results raise the possibility that increases in airway SMC number rather than, or in addition to, chronic inflammation contribute to the persistent AHR detected in this model.

asthma; airway wall remodeling; growth; airway mechanics; stereology

AIRWAY REMODELING PROCESSES in asthma may play an important role in modulating airway mechanics and provide an explanation for at least part of the airway hyperresponsiveness (AHR) seen in asthma (24). The remodeling comprises epithelial denudation, inflammatory cell infiltration, goblet cell metaplasia, subepithelial fibrosis, angiogenesis, and smooth muscle hyperplasia and hypertrophy in the airway wall of asthmatic subjects (19), of which increases in the volume of airway smooth muscle (ASM) appear to have the most significant impact on AHR. Limited numbers of studies in which the number of smooth muscle cells (SMCs) in airways from asthmatic and healthy individuals were counted have shown either that there was an increase in SMC number in the airways of asthmatic subjects compared with control airways (12, 18) or that there was no increase in asthmatic SMC number (35). The merit of the use of stereological techniques in the latter study notwithstanding, the use of airways from smokers as controls confounded the interpretation, as it is well established that chronic obstructive pulmonary disease is associated with airway wall remodeling and increases in the volume of ASM 

Increases in the volume of ASM have been detected in sensitized and antigen-challenged rats (10, 33, 37) and cats (25, 26). However, it is still unclear whether the increased volume of muscle detected in these studies is due to hyperplasia or to hypertrophy of SMCs or to an increased volume of extracellular matrix. Recent studies examining DNA synthesis are strongly suggestive of ASM proliferation (28, 30–32), but this interpretation has not been formally tested.

We have used the ovalbumin (OA)-challenged Brown Norway (BN) rat, in which chronic OA challenge is an established stimulus for airway wall remodeling (9, 20, 28, 30–33), to examine whether OA challenge resulted in increased SMC number in large airways. In addition, the effects of FCS alone and when combined with OA were examined, as FCS is known to enhance the proliferation of individual cell types that contribute to the remodeling response. It was anticipated that such exposure to FCS would increase the SMC number to a greater extent than OA alone. The use of FCS would, therefore, facilitate exploration of any relationship between SMC number and airway reactivity over a greater range of SMC number than generated by the standard chronic OA challenge.

To investigate the SMC hyperplasia, we have applied a new approach to SMC counting with a simple, direct, and efficient method, instead of the tedious methods of SMC counting used in previous studies. With the use of the Cavalieri and the optical dissector...
methods (4), the total number of airway SMCs in the main bronchus has been estimated. Mast cell numbers were enumerated because increased numbers have been identified in the muscularis of asthmatic subjects (2), and many mast cell mediators are potentially significant in initiating airway SMC proliferation.

OA challenge of BN rats resulted in a modest level of AHR, but there was no increase in the number of airway SMC in the left main bronchus. However, the incorporation of FCS into the antigen challenge protocol resulted in significant increases in SMC number and AHR that persisted for at least 7 days after the final OA and FCS exposure.

**METHODS**

**Sensitization and OA exposure.** In the first series of experiments, 12 male BN rats (180–200 g, Animal Resource Centre) were sensitized by a single 1-mL intraperitoneal (ip) injection of OA (1 mg/ml in 100 mg/ml aluminium hydroxide in sterile saline). In addition, each rat received a 0.5-mL ip injection of the adjuvant Bordetella pertussis containing 6 × 10⁷ heat-killed organisms. Fourteen days after sensitization, the rats were divided into two groups: one group was exposed to an aerosol of 5% (wt/vol) OA generated by a Hudson nebulizer (driven by a flow rate of 8 l/min of compressed air) for 15 min on three occasions with an interval of 5 days between each exposure (OA group, n = 6), whereas the other group was exposed to an aerosol of saline of the same duration (saline group, n = 6). In the second series of experiments, 32 male BN rats (220–290 g) were sensitized as described above. Fourteen days after sensitization, the rats were divided into four groups: one group was exposed to an aerosol of saline for 15 min on four occasions with an interval of 5 days between each exposure (saline group, n = 8), whereas the other three groups were exposed to an aerosol of FCS (10% vol/vol; FCS group, n = 7), OA (5% wt/vol; OA group, n = 7), or FCS and OA (FOA group, n = 6) for the same duration. The OA sensitization and challenge protocol was modified from that used by Sapienza et al. (33). The experiments described in this manuscript were conducted in compliance with the guidelines of the National Health Medical Research Council of Australia on animal experimentation.

**Assessment of airway responsiveness.** Airway reactivity to ACh was assessed in vivo 24 h (first series of experiments) or 7 days (second series of experiments) after the last aerosol challenge. The rats were anesthetized with an ip injection of pentobarbital sodium (12 mg/kg) and ethyl carbamate (1 g/kg). A tracheal cannula, through which the rat was mechanically ventilated with room air, was inserted via a tracheotomy. Cannulas were also inserted into the external jugular vein for drug administration and into the carotid artery for blood pressure monitoring. The rat was then placed in a whole body plethysmograph and mechanically ventilated (10 ml/kg, 60 breaths/min) (rodent ventilator, UGO Basile). The transrespiratory pressure was measured with a differential pressure transducer (Validyne) with one port attached to the interior of the plethysmograph and the other port attached to the intratracheal cannula. The airflow rate was measured with a pneumotachograph. The respiratory resistance and compliance were calculated on-line with a Buxco pulmonary mechanics analyzer (Buxco Electronic) based on the principles of Amdur and Mead (1), and the outputs were displayed on a Macintosh LC III monitor (Apple) by using MacLab software (AD Instruments). After surgery, an initial dose of gallamine triethiodide (8 mg/kg) was administered intravenously to inhibit spontaneous respiratory movements, and further doses of 4 mg/kg were administered as required. After stabilization of cardiorespiratory parameters (15–20 min), the rat was given ACh intravenously at an initial dose of 25 μg/kg and then in increasing doses by doubling up to 400 μg/kg to obtain a response of >100% increase over baseline. The response was measured as the peak increase above the baseline immediately before ACh administration. The dose required to increase respiratory resistance by 100% (PC100) was estimated by log-linear interpolation of dose-response curves from individual animals. After assessment, the rats were killed by an overdose of anesthetic.

**Tissue preparation.** Fixation in situ with 4% paraformaldehyde in 0.1 M phosphate buffer was achieved by perfusion via a tracheal cannula and via an abdominal aorta cannula. The pressure for tracheal infusion was 25 cmH₂O and for the arterial perfusion was 100 mmHg. After 3 min of in situ fixation, the lung was removed from the rat and immersed in the same fixative for 24 h. Samples from the right main bronchus and lung were paraffin embedded. The paraffin sections (4-μm thickness) were used for hematoxylin and eosin and toluidine blue staining. The left main bronchus was used for stereological study of ASM. The specimen was defined anatomically as beginning at the tracheal bifurcation and continuing until the next bifurcation. The dissected left main bronchus was dehydrated through a series of increasing concentrations of ethanol. The sample was infiltrated with catalyzed ImmunoBed A solution and embedded with a mixture of 1 part of ImmunoBed B and 25 parts of catalyzed immunoBed A (Polysciences, Warrington, PA). The resin-embedded specimens were serially sectioned at 30 μm (Fig. 1).

**Histological staining and enumeration of eosinophils and mast cells.** The 4-μm paraffin sections of 4% paraformaldehyde-fixed bronchial airway were stained with hematoxylin and eosin or toluidine blue by using standard techniques, and mast cells and eosinophils were enumerated by using Image Analysis software (see below). The 30-μm sections of resin-embedded right bronchi were stained with hematoxylin for 60 min and eosin for 15 min to allow permeation of the stains through the plastic resin. SMCs were enumerated by using stereological techniques as described below in detail.

**Enumeration of eosinophils and mast cells.** Eosinophil and mast cell numbers within the airway wall of the left bronchus were enumerated by using standard image analysis techniques. Briefly, the number of eosinophils (eosin-positive granulated cells) and mast cells (metachromatic appearance in toluidine blue-stained sections) were counted in a complete airway wall section from each animal in each of the subject groups with the aid of a light microscope (Leica DMIRB). The number of positive cells was normalized to the respective sample area (area of bronchus wall) by using a calibrated image analysis system comprising an RGB video camera (Hitachi) coupled to Image Pro Plus 4.0 image analysis software (Media Cybernetics). The cell numbers are expressed as the number of positive cells per millimeter square of bronchus wall.

**Stereology.** The bronchial wall is composed of four layers: mucosa (epithelium, basement membrane, lamina propria), submucosa, cartilage, and adventitia (3). The bronchial wall area measured in this stereological study did not include the adventitial layer because of the difficulty in defining its outer border on the sections of bronchial wall. A systematic, random sampling of thick (30-μm) sections was used to evaluate SMC number (7, 17). The bronchus was exhaustively sectioned at 30-μm thickness by using a Reichert-Jung 1150 Autocut (Nussloch, Germany) fitted with...
a glass ralph knife. Beginning with a random start, every 20th section was sampled, mounted on a poly-L-lysine-coated glass slide, stained with hematoxylin (60 min) and eosin (15 min), and viewed by microscopy. The image was displayed on a high-resolution monitor at a final magnification of 1,100 by a video camera, and a series of systematic fields were selected with the aid of an electronic stepping stage. The "optical dissector" method was employed to count SMCs (16) within the acceptance area of a three-dimensional (3D) computer-generated unbiased counting frame (or else brick) of 275-μm² area and 10-μm depth. The topmost plane of the optical dissector was positioned at a minimum of 10 μm below the cut surface of the section, and all of the SMC nuclei that came into focus within a subsequent depth of 10 μm were counted. For each bronchus, a minimum of 10 sections was sampled and 150–200 SMCs counted, to accurately determine the total number of SMCs (10, 17).

The numerical density of SMC in the bronchial wall was calculated by dividing the number of SMCs counted by the volume sampled such that

\[ N_v = \frac{\sum N_{smc}}{\sum P_{d} \times V(P)} \]

where \( N_v \) is SMC density, \( \sum N_{smc} \) is total number of SMC counted, \( \sum P_{d} \) is total number of points sampled, and \( V(P) \) is the volume associated with each point.

The Cavalieri method was used to estimate the volume of the bronchus as defined for the optical dissector. First, the total cross-sectional area of the previously sampled bronchi sections was determined by point counting. The volume was then calculated by using the following equation

\[ V_{bron} = \frac{\sum P_{d} \times A(P) \times T}{\text{total number of sections/number of sections sampled}} \]

where \( V_{bron} \) is volume of bronchus, \( \sum P_{d} \) is total number of points overlying the bronchial wall in all sampled sections, \( A(P) \) is the area of each test grid (point), and \( T \) is thickness of each section.

The \( N_{smc} \) in the bronchus was calculated by multiplying the \( N_v \) by the \( V_{bron} \), where \( N_{smc} = N_v \times V_{bron} \).

Materials. The following chemicals were used: ACh (BDH), aluminium hydroxide (BDH), Bordetella pertussis (CSL), ethyl carbamate (AJAX, Sydney, Australia), gallamine triethiodide (Flaxedil, May & Baker), ImmunoBed A (Polysciences), ImmunoBed B (Polysciences), Nembutal (Boehringer Ingelheim), OA (Grade II, Sigma Chemical, St. Louis, MO), paraformaldehyde (Probing & Structure), poly-L-lysine (Sigma Chemical), and toluidine blue (Sigma Chemical).

Statistical analysis of results. Results are presented as grouped data from \( n \) rats and are expressed as means ± SE; \( n \) represents the number of rats. Student’s unpaired \( t \)-test was used to determine whether there were significant differences between pairs of means. In some cases, a two-way ANOVA was used to investigate whether there was an effect of OA or FCS treatment and whether there was an interaction between the two variables. All statistical analyses were performed by using GraphPad Prism for Windows (version 2.01). In all cases, probability levels \( < 0.05 \) (\( P < 0.05 \)) were taken to indicate statistical significance.

RESULTS

Immediate response to OA. Most of the animals that were exposed to an aerosol of OA developed an immediate response within 2–5 min. The responders sneezed and showed increased amplitude of breathing movements and wheezing. Signs of obstruction resolved quickly after the termination of OA exposure. Saline-exposed rats did not display these signs.
Baseline respiratory resistance. There was no significant difference in weight (251 ± 1 g for saline vs. 254 ± 2 g for OA) or baseline respiratory resistance (0.44 ± 0.04 cmH₂O·m⁻¹·s for saline vs. 0.41 ± 0.04 cmH₂O·m⁻¹·s for OA) between sensitized, saline-challenged and sensitized, OA-challenged animals that had their airway mechanics assessment 7 days after the last aerosol challenge (Table 1). The baseline respiratory resistance values for FCS- and OA-treated animals were significantly different from those of saline-treated animals (P < 0.05, two-way ANOVA), but there was no significant interaction between FCS and OVA treatment (P > 0.05, two-way ANOVA) (Table 1).

Airway responses to ACh. In the first series of experiments in which airway mechanics were assessed 24 h after the last aerosol challenge, both sensitized saline-challenged and sensitized OA-challenged animals showed a dose-dependent increase in respiratory resistance to intravenous ACh. However, OA-challenged rats had significantly greater responses to ACh at doses of 100 and 200 μg/kg compared with sensitized saline-challenged rats (Fig. 2). The PC₁₀₀ was significantly lower for OA-challenged animals [log(PC₁₀₀): 2.12 ± 0.02] than for the saline group [log(PC₁₀₀): 2.48 ± 0.07; P < 0.01].

In the second series of experiments in which airway mechanics were assessed 7 days after the last aerosol challenge, the PC₁₀₀ was significantly lower in FCS-treated than in saline-treated animals (P > 0.05, two-way ANOVA) but was not different in animals treated with OA alone (P > 0.05, two-way ANOVA) (Table 1). In addition, two-way ANOVA revealed that there was no interaction between FCS and OA (P = 0.6179).

Airway inflammation. The OA challenge protocol used in both series of experiments produced an obvious airway inflammation (Fig. 3). In the first series of experiments, marked eosinophil infiltration and lymphocytic nodules in the airways and large mononuclear cell-dominated, mixed cell nodules in the alveolar spaces were observed 24 h after exposure to the last of three OA challenges (each separated by 5 days). In saline-challenged animals, there was no consistent inflammatory cell infiltration, although isolated lymphocytic nodules and a small number of eosinophils were observed in some specimens (Fig. 3). Eosinophil number increased significantly (P < 0.05, unpaired t-test) from 22 ± 6 (number/mm² bronchus wall area) in saline-treated rats to 362 ± 102 in OA-exposed rats. In contrast, mast cell numbers in saline-exposed rats (48 ± 7) were not different from those in OA-exposed rats (63 ± 18). Moreover, the distribution of mast cells did not appear to be affected by OA challenge (data not shown).

The degree of inflammation in airways harvested 7 days after the last exposure to OA appeared to be less than that observed 24 h after the final challenge (Fig. 3). However, the eosinophilia in response to OA was not statistically different from that observed in tissues harvested 24 h after the last OA exposure (Table 2). Furthermore, FCS alone had no significant effect on eosinophil number but appeared to reduce the response to OA (Table 2). Mast cell numbers were unchanged (P > 0.05) by OA, FCS, or FOA exposure when measured 7 days after the last aerosol exposure (Table 2).

SMC number. In the first series of experiments in which airway tissue was harvested 24 h after the last challenge, OA aerosol challenge had no effect on SMC number (saline exposed, 5.17 ± 0.29 × 10⁵; OA exposed, 5.54 ± 0.47 × 10⁵) or left main bronchus volume (saline exposed, 5.98 ± 0.23 mm³; OA exposed, 6.41 ± 0.28 mm³). However, in animals that had airway mechanics assessed 7 days after the last aerosol challenge, OA alone and the combination of FCS and OA treatment increased SMC numerical density (P < 0.05, two-way ANOVA) and the total number of SMCs in the left main bronchus (P < 0.001, two-way ANOVA) (Table 3). Although there was a significant interaction
between FCS and OA with respect to increases in SMC numerical density ($P = 0.0314$, two-way ANOVA) and total number of SMC ($P < 0.0001$, two-way ANOVA) (Table 3), FCS alone had no effect on either of these measures of remodeling. The combination of FCS and OA treatment increased bronchus volume ($P < 0.05$, two-way ANOVA), but neither stimulus alone had an effect ($P > 0.05$, two-way ANOVA) (Table 3).

**DISCUSSION**

In this study, the existence of smooth muscle hyperplasia was investigated in a well-established model of antigen-induced airway wall remodeling in OA-sensitized BN rats. Previous studies have established an increase in the mass of ASM and an increase in the fraction of cells incorporating bromodeoxyuridine (BrdU), a marker of cells in, or having been in, S phase (28, 30–32). These observations suggest, but do not establish, that the increase in ASM mass is at least partly the result of ASM hyperplasia, with hypertrophy and increased extracellular matrix also having the potential to increase the volume of bronchus wall occupied by smooth muscle. The number of SMC in the bronchus was investigated by using methods of unbiased stereology to directly ascertain whether the reported increased volume of smooth muscle observed in BN rat airways may be explained by an increase in SMC number. There was no increase in SMC number in the left main bronchus of sensitized BN rats that were exposed to three aerosols of OA, despite increased airway reactivity and inflammation. However, when animals were exposed to four aerosols of OA or when FCS was incorporated into the OA challenge protocol, there was a significant increase in SMC number. The reasons for the difference in the outcome between three and four OA exposures may relate to the additional time for remodeling to occur in the latter protocol (13 days extra). Although a number of studies report increased ASM mass after only three challenges (28, 30–32), the differences in the efficiency of delivery of the OA challenge in different protocols precludes meaningful direct comparison of the extent of OA challenge required for remodeling.

The BN rat model of OA-induced inflammation has been used extensively to evaluate the mechanisms underlying airway inflammation and AHR. Sensitization alone has no effect on airway responsiveness and does not elicit measurable airway inflammation (14, 15, 20, 29, 36). A single OA challenge appears to be adequate to induce transient AHR, but multiple OA aerosol challenges are required for persistent AHR (15, 33). Cysteinyl leukotrienes (31) and endothelin (30)

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**Table 2. Eosinophil and mast cell numbers in the right main bronchus of sensitized BN rats exposed to aerosols of saline, OA, FCS, and FOA**

<table>
<thead>
<tr>
<th>Group</th>
<th>Eosinophils, no./mm²</th>
<th>Mast cells, no./mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>29 ± 9</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>FCS</td>
<td>65 ± 21</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>OA</td>
<td>358 ± 119*</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>FOA</td>
<td>79 ± 18</td>
<td>15 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE. *$P < 0.05$, unpaired $t$-test, compared with saline-treated rats.
antagonists reduce the SMC DNA synthesis induced by repeated OA exposure without reducing the AHR in this model. In the present study, sensitized BN rats developed a persistent AHR (7 days) after a combination of OA and FCS challenges, but not after OA alone, even though OA alone was associated with a transient AHR. There was an obvious eosinophilic airway inflammation 24 h after the last OA challenge that had not resolved after 7 days. Recently, Palmans et al. (27) showed that, after 2 wk of OA exposure, there was an increase in AHR and eosinophilic inflammation, but repeated OA exposure for 4–12 wk was accompanied by a resolution of the AHR, despite persistent eosinophilic inflammation. Thus eosinophilic airway inflammation may not be the only factor regulating the development of AHR. Studies using different protocols for OA exposure have provided evidence consistent with a dissociation of eosinophilia and AHR by showing that AHR persists beyond the eosinophilic response. Repeated, but not single, OA challenge of BN rats induced a persistent AHR at a time when eosinophilic airway inflammation was resolving (15). Cui et al. (9) also demonstrated that 9 wk of antigen challenge resulted in AHR without concurrent eosinophilia. Thus, whereas the current evidence does not preclude a role for eosinophils in either airway wall remodeling or AHR, persistent eosinophilia does not appear to be required for persistent AHR in the BN rat model. Recent studies in atopic and patients with mild asthma support the notion that eosinophils alone are not responsible for allergen-induced AHR or the late asthmatic response (6, 22). Systemic administration of recombinant human interleukin-12 to patients with mild allergic asthma caused a decrease in blood and sputum eosinophil numbers (6). Intravenous administration of an interleukin-5-blocking antibody to patients with allergic asthma caused a marked suppression of the increase in blood and sputum eosinophil numbers after allergen challenge but did not protect against the allergen-induced late asthmatic response or the increase in airway responsiveness to histamine (22). T cells adoptively transferred from OA-sensitized rats confer increased reactivity on OA-challenged recipient rats (23). The role of T cells in AHR in asthma has yet to be defined.

A digitized tracing method has been widely used to show that airway SMC volume is increased in asthmatic airways. Only three studies have attempted to resolve the contributions of smooth muscle hyperplasia and/or hypertrophy to this increase in SMC volume in asthmatic airways by directly counting the number of SMCs (12, 18, 35). Two of these studies showed a threefold increase in SMC number in the large airways of human asthmatic subjects compared with healthy individuals (12, 18), whereas the third failed to find an increase in SMC number, possibly because of inclusion in the “nonasthmatic” control group of tobacco smokers and patients with emphysema (35). Airways from smokers and chronic obstructive pulmonary disease patients may be thickened and show SMC volume increases (5, 21). Ebina and colleagues (12) used 3D reconstruction of a series of sections from control and asthmatic subjects to demonstrate hyperplasia as well as hypertrophy of ASM in asthma. Asthmatic patients were classified into two types: type I asthmatic airways showed SMC hyperplasia only in the larger bronchi, and there was no SMC hypertrophy in either the large or small airways; type II asthmatic airways, on the other hand, showed only mild hyperplasia in the larger bronchi, but SMC hypertrophy was evident in large and small airways.

We have used a new approach to SMC counting by a combination of unbiased, systematic, random-start sampling and the use of an unbiased probe: the optical dissector under high magnification. This sampling method is unbiased because there is no design-derived error (8, 17). The optical dissector method allows the use of thick sections and the counting of SMC directly in a series of 3D counting frames. This approach is much simpler and more efficient than the 3D reconstruction method of Ebina et al. (12), although similar principles are applied. In addition, tissue sections were embedded in plastic rather than paraffin, because tissue shrinkage is <4% in plastic sections, but up to 14% in paraffin sections (35).

Sensitized BN rats that had been exposed to four aerosols of OA showed a significant increase in SMC number in the left main bronchus compared with sensitized, saline-challenged animals. Interestingly, the incorporation of FCS into the antigen-exposure protocol resulted in a greater increase in SMC number than that observed in animals treated with OA alone. FCS is mitogenic for cultured airway SMCs (34). Thus it was not surprising to find an increase in SMC number in FCS-treated animals and synergy between FCS and OA. In addition, the bronchus volume and SMC numerical density were also significantly increased in animals treated with FOA. These results are consistent with earlier studies showing increased smooth muscle area in antigen-challenged BN rats: Sapienza et al. (33) demonstrated a twofold increase in the quantity of ASM in small, medium, and large airways of OA-challenged rats; Cui et al. (9) showed that chronic

### Table 3. Smooth muscle cell numerical density, left main bronchus volume, and total number of smooth muscle cells in the left main bronchus of sensitized BN rats exposed to aerosols of saline, OA, FCS, and FOA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Smooth Muscle Cell Numerical Density, no./mm³</th>
<th>Vbron, mm³</th>
<th>Nsmc, total number of smooth muscle cells in left main bronchus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>53,515 ± 2,948</td>
<td>5.91 ± 0.03</td>
<td>316,349 ± 16,942</td>
</tr>
<tr>
<td>FCS</td>
<td>55,445 ± 3,464</td>
<td>6.29 ± 0.28</td>
<td>350,755 ± 31,361</td>
</tr>
<tr>
<td>OA</td>
<td>70,327 ± 6,732*</td>
<td>6.91 ± 0.66</td>
<td>468,577 ± 32,116*</td>
</tr>
<tr>
<td>FOA</td>
<td>101,044 &lt; 11,109*</td>
<td>8.83 ± 0.79*</td>
<td>862,897 ± 69,215*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Vbron, volume of left main bronchus; Nsmc, total number of smooth muscle cells in left main bronchus. *P < 0.05, unpaired t-test, compared with saline-treated rats.
trimellitic anhydride (occupational allergen) exposure increased the thickness of smooth muscle in the small airways in sensitized BN rats; Wang et al. (37) also demonstrated an increase in ASM volume in large airways (internal perimeter > 2 mm) of OA-challenged rats; Panettieri et al. (28), using BrdU incorporation, indicated that DNA synthesis increased in SMCs after three OA challenges. Similarly, Salmon et al. (32) showed that repeated OA exposure increased the fraction of BrdU-positive ASM in the BN rat model. Our findings suggest that the increase in ASM area is explained in part by an increase in SMC number, but we have not estimated the area/volume of airway wall occupied by smooth muscle. A recent study by Palmans et al. (27) showed that the area of smooth muscle around the airways did not change in OA-challenged BN rats, despite an increase in the total wall area of small, medium, and large airways in sensitized rats exposed to OA for 2 wk. It remains possible that SMC number increases without detectable increases in area occupied by ASM.

Increased reactivity 24 h, but not 7 days, after the final OA challenge in our chronic exposure study suggests that inflammatory factors rather than structural changes are key determinants of this acute AHR. Incorporation of FCS into the OA challenge protocol (FOA) resulted in a substantial increase in SMC number and a modest, persistent AHR. However, the AHR in FOA-treated animals was largely associated with an interaction between FCS and OA, because neither treatment alone showed marked increases in responsiveness. Interestingly, the persistent eosinophilia seen 7 days post-OA challenge was diminished by the addition of FCS to the challenge aerosol, providing further evidence of a dissociation between eosinophilia and persistent AHR. In animals treated with FOA, there was an increase in SMC number and AHR, raising the possibility that structural rather than inflammatory mechanisms may contribute to the persistence of AHR after repeated FOA challenge in this model. Nevertheless, it is also possible that FCS induced a reactivity change that was independent of the increase in SMC number, as FCS alone increased reactivity without influencing SMC number.

In summary, stereological methods were used to study ASM hyperplasia in sensitized antigen-challenged BN rats. There was no hyperplasia of SMC in the left main bronchus in animals exposed to three aerosols of OA, despite an acute increase in AHR and airway inflammation. However, sensitized rats that received four aerosols of OA showed a significant increase in SMC number that was further increased when FCS was incorporated into the OA challenge protocol. These animals also displayed AHR, but this effect could be attributed to the actions of FCS. Thus chronic exposure to OA can cause acute reactivity changes, but additional stimuli are required for persistent changes in reactivity in these relatively short treatment protocols. Compared with traditional two-dimensional morphological studies, modern stereology provides a new, simple, and efficient approach to obtaining precise and reproducible information in a 3D volume. Future applications of this technique to the studies of airway remodeling will advance our knowledge of the structure-function relationship in airways that have undergone remodeling.

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