Asthma is a chronic inflammatory condition. When asthma is allergen driven it is a Th2-type disease and is characterized by features that include reversible airway obstruction, chronic inflammation, airway hyperresponsiveness (AHR), and airway remodeling. Airway remodeling comprises structural changes in virtually all cells of the airway wall. Prominent among these changes are epithelial proliferation (31) and denudation (21), reticular basement membrane thickening (17), goblet cell metaplasia (19), subepithelial fibrosis (32), increase in airway smooth muscle (ASM) mass (11), and mucus gland hyperplasia and hypertrophy (7). The mechanisms of airway remodeling are complex and likely involve lipid mediators, cytokines, chemokines, and growth factors released from both inflammatory and structural cells (12).

AHR is a reflection of the enhanced capacity of the airways to respond to bronchoconstrictors and as such may be predominately a reflection of the ability of the ASM to overcome intrinsic impedances to airway narrowing (36). Although it seems intuitively obvious that an increase in ASM mass should lead to AHR, data supporting this hypothesis have been elusive. Further uncertainty about the relationship between remodeling of ASM and AHR is based on the fact that a change in phenotype with loss of contractile proteins may follow hyperplasia (20, 26). Additionally, the degree of remodeling and changes in ASM properties may not be uniform throughout the airway tree (26). Given the current view that airway remodeling, and ASM remodeling in particular, has key therapeutic importance we felt that it was important to assess whether AHR is dissociable from ASM remodeling. Alternative mechanisms for AHR have been proposed. For example, AHR may reflect geometric effects of remodeling of tissues internal to the ASM itself (43) or through encroachment on the lumen by mucus. Subepithelial fibrosis has been argued to enhance airway narrowing by impeding airflow (44). Fibrosis has also been proposed to enhance AHR by stiffening the airway and reducing the effects of the cyclical breathing movements on the stretching of ASM associated with these movements (13, 36).

To investigate the relationship between ASM growth and the alterations in airway responsiveness to methacholine (MCh), we used a well-characterized rat model of “allergic asthma” in which reproducible remodeling of ASM occurs after repeated inhalational challenges with allergen (33, 34, 38, 46). Using morphometric techniques we identified the site of ASM remodeling induced by allergen challenges and compared it with the site of AHR to MCh. We also quantified the changes in goblet cell number, the change in airway area, and the thickness of the airway tissues inside the inner aspect of the ASM bundles because of the postulated importance of these aspects of remodeling to AHR (27). To determine the site within the respiratory tree responsible for AHR we applied the constant-phase model of respiratory mechanics to partition the changes in mechanics following aerosol challenge with MCh that were attributable to either the large airways or the peripheral compartments of the lung. We also compared our findings with the more frequently used single-compartment model to evaluate respiratory system resistance (Rrs) and elastance (Ers) responses. As an additional test of the mechanistic link between ASM growth and AHR we administered a topical corticosteroid, budesonide (Bud), before allergen challenges, reasoning that the drug treatment might affect AHR and remodeling differently. AHR also follows a single allergen challenge during which time ASM growth does not have time to occur. Inflammatory mechanisms and, in particular, the cytokine IL-13 have been implicated in this form of AHR (39, 42). We were interested in examining whether the changes in mechan-
ical properties induced under these circumstances were similar to those induced by repeated allergen challenges accompanied by ASM remodeling. We performed a multiplex assay of cytokines to examine the relationship between the AHR following repeated allergen challenge and potential biomarkers present in bronchoalveolar lavage (BAL) fluid.

**METHODS**

**Protocol for sensitization and challenge.** Male Brown Norway (BN) rats (SsN substrain) (Harlan UK) were sensitized subcutaneously with 1 mg of ovalbumin (Ova) and 100 mg of aluminum hydroxide dissolved in 1 ml of phosphate-buffered saline (PBS) (38). Concurrently, the rats were injected intraperitoneally with $2 \times 10^9$ Bordetella pertussis heat-killed bacteria (provided by T. Issekutz, Dalhousie University, Halifax, NS, Canada) (38). Aerosol challenges, with either 5% Ova or the PBS control, were performed in endotracheally intubated animals breathing spontaneously under light pentobarbital anesthesia. The endotracheal tube was placed in a small Plexiglas box into which aerosol was introduced from a Hudson nebulizer with an output of 0.15 ml/min. Two protocols of allergen challenge were followed. In the first protocol rats were challenged once and studied at 24 h after the challenge. In the second protocol, rats were challenged a total of three times with Ova, or its control (PBS), at 5-day intervals (38) and studied 48 h or 1 wk after the final challenge (Supplemental Fig. S1).1 The study protocols were approved by the Animal Care Committee of McGill University.

**Treatment with budesonide.** Fourteen days after Ova sensitization as described above, rats were administered either 0.1 mg/kg Bud or its vehicle, saline, intranasally 24 h and 1 h before Ova challenge. Animals in an additional group were killed at 1 wk after three challenges to test whether AHR, ASM, and epithelial cell remodeling persisted at this time point.

Bud (0.1 mg/kg) was prepared from a suspension manufactured for inhalation (Pulmicort Nebuamp, Astra Zeneca, Mississauga, ON, Canada) and was administered intranasally in 100 μl of saline.

**Assessment of lung deposition of intranasally administered suspension.** Evans blue dye (500 μg) was delivered intranasally in the same manner as Bud. The animals were lightly anesthetized with isoflurane during the delivery. The animals were killed 1 h later with anesthetic overdose. The trachea and lungs were removed, and Evans blue dye was extracted with formamide in a 60°C water bath for 24 h. Optical density was measured at 620 nm with a spectrophotometer. Extracted Evans blue was quantified on a standard curve. We determined that the signal was developed with Vector Red. The area of staining to inflammatory mediators we performed a multiplex cytokine analysis on the first 5 ml of BAL samples retrieved at the 48 h time point in the PBS, Ova, and Bud+Ova groups, using a multiplex rat cytokine kit (LINCOPLEX, LINCO Research/Millipore, St. Charles, MO) as described previously (5) and after concentrating the samples 20-fold. The following cytokines/chemokines were measured: eotaxin, regulated on activation normal T-expressed and presumably secreted (RANTES), growth-related oncogene KC (GRO KC), IL-1α, IL-1β, IL-13, IL-4, IL-5, IL-6, and macrophage inflammatory protein-1α (MIP-1α).

**Histology.** For the assessment of remodeling the left lungs were fixed overnight in formalin at 25 cmH$_2$O and were paraffin embedded. Histological slides were prepared from 5-μm mid sagittal and parahilar sections to sample medium and small airways and large airways, respectively. The tissue sections were stained for smooth muscle-specific α-actin (α-SMA) to assess the increase in immunoreactive area (30). Isotype controls were performed and showed no staining, confirming the specificity of anti-α-SMA antibody. Airways of ≥1-mm basement membrane perimeter (PBM) were considered as medium and/or large airways and those of <1-mm PBM as small airways (30). In brief, sections were stained with a mouse monoclonal antibody to α-SMA (clone I4A4; Sigma-Aldrich) as well as a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories). The signal was developed with Vector Red. The area of ASM was traced with a camera lucida side arm microscope attachment and was digitized with commercial software (SigmaScan Pro 5, Ashburn, VA). The area of ASM was normalized for airway size by dividing by the square of the PBM.

To assess goblet cell hyperplasia, sections were stained with periodic acid Schiff (PAS) (Sigma Chemical). The quantitative assessment was performed by counting the number of PAS positively stained cells and normalizing by the PBM.

**Bronchoalveolar lavage and assessment of inflammation.** BAL (5 × 5-ml aliquots of PBS) was performed after the assessment of airway responses. Cells were pelleted by centrifugation from the five aliquots and resuspended in PBS for cell counts. Cytosplasts were prepared with a cytospin centrifuge (Shandon Cytospin 4 cytospincentrifuge, Thermo Scientific, Waltham, MA) and were stained with Diff-Quik Stain Set (Dade Behring, Newark, DE) to assess inflammation.

**Cytokine analysis.** To assess the relationship of AHR and remodeling to inflammatory mediators we performed a multiplex cytokine analysis on the first 5 ml of BAL samples retrieved at the 48 h time point in the PBS, Ova, and Bud+Ova groups, using a multiplex rat cytokine kit (LINCOPLEX, LINCO Research/Millipore, St. Charles, MO) as described previously (5) and after concentrating the samples 20-fold. The following cytokines/chemokines were measured: eotaxin, regulated on activation normal T-expressed and presumably secreted (RANTES), growth-related oncogene KC (GRO KC), IL-1α, IL-1β, IL-13, IL-4, IL-5, IL-6, and macrophage inflammatory protein-1α (MIP-1α).
followed by a Bonferroni correction. A *P* value of <0.05 was considered significant.

A linear discriminant analysis (LDA) was performed for the cytokines/chemokines assessed in the BAL fluid with the multiplex kit, and statistical analysis was performed with R (R Foundation for Statistical Computing, Vienna, Austria). The LDA was performed to determine whether the cytokine results in the various treatment groups provided sufficient information to discriminate or classify treatment groups as separate from one another (Supplemental Fig. S3). The first linear discriminant function discriminated the Ova group from the PBS and Bud/Ova groups. The second linear discriminant function discriminated the PBS group from the Bud/Ova group.

**RESULTS**

**Allergen-induced changes in airway responsiveness to methacholine: single-compartment model.** At 24 h after a single Ova challenge, AHR was detectable as exaggerated responses in Ers to MCh challenge, (Fig. 1A). No significant changes in Rrs were observed (Fig. 1B). At 48 h after three Ova challenges, the animals had a higher Rrs to aerosolized MCh (Fig. 1D) and a significantly higher Ers than the PBS-challenged animals (Fig. 1C). The increase in responsiveness to MCh was no longer present at 1 wk after the three Ova challenges (data not shown). AHR after a single Ova challenge was inhibited by Bud administered before Ova challenge (*P* < 0.05, Fig. 1A).

**Allergen-induced changes in airway hyperresponsiveness: constant-phase model.** To resolve the site of airway responsiveness to the large conducting airways or the more peripheral lung, we analyzed responses to MCh with the constant-phase model. Ova challenge did not alter the responses in RN following MCh challenge. Neither a single Ova challenge (Fig. 2A) nor repeated Ova challenges (Fig. 2B) augmented the response in
Airways. In the Ova group versus the Ova group and the Bud challenged group and the Bud challenged group had an increased H compared with the PBS group in response to MCh (P < 0.05, Fig. 4A) and this change was not inhibited by Bud (Fig. 4A). The H parameter in the Ova-challenged animals was not elevated at either 48 h (Fig. 4B) or 1 wk (data not shown) after three Ova challenges.

Bronchoalveolar lavage inflammatory cells. At 24 h after a single challenge, the Ova-challenged animals had significantly higher eosinophil numbers than the PBS-challenged animals, which was not reduced by Bud (P < 0.05, Fig. 5A). At 48 h after three Ova challenges eosinophilia was present and was not reduced by Bud (Fig. 5B). While the total numbers of cells in the various treatment groups were reduced at 1 wk compared with the 48 h time point, the total number of cells and macrophages in the Ova-challenged animals were still significantly higher compared with the PBS-challenged animals (P < 0.05; data not shown).

Airway smooth muscle growth. We determined the extent and site of remodeling of ASM after three Ova challenges at 48 h and 1 wk (Fig. 6B and data not shown). At 48 h, the ASM area in the Ova animals was significantly greater than in PBS animals in both the central airways (P < 0.05) and peripheral airways (P < 0.05). Bud inhibited the increase in ASM area induced by Ova challenge in both central and peripheral airways (P < 0.05, Fig. 6B). The increased ASM was maintained for at least 1 wk in the peripheral airways (P < 0.05) and showed a trend for persistence in the central airways (P = 0.07; data not shown).

Total airway wall area and airway wall thickening. In the central airways, the total airway wall area was significantly increased in the repeated OVA-challenged animals compared with the control animals (P < 0.05, Fig. 7A). In the peripheral airways, a similar pattern was observed (P < 0.01, Fig. 7A). Bud inhibited the increased total airway wall area in the peripheral airways (P < 0.05) but did not reduce central total airway wall area (Fig. 7A). There were no significant observable differences in airway wall thickening inside the ASM bundles after repeated Ova challenges (Fig. 7B).

Goblet cell hyperplasia. The Ova animals had an increased number of goblet cells/PBM in the central airways compared with the PBS-challenged control animals, which was attenuated by Bud (Fig. 8B, P < 0.05). The goblet cell numbers were persistently elevated in the central airways by 1 wk after three Ova challenges but increased even further in the peripheral airways compared with 48 h after repeated allergen challenges (P < 0.0001, Fig. 8B and data not shown).

Cytokine/chemokine analysis. Given the apparently stronger relationship between inflammation and AHR compared with remodeling and AHR, we performed a LDA on mediators in BAL samples collected 48 h after three challenges. From the plot of the first two discriminant functions, we found that the first function clearly discriminated between the PBS-challenged group and the Bud+Ova group versus the Ova group (Supplemental Fig. S2). Meanwhile, the second function discriminated between the Bud+Ova group and the PBS group.
The cytokines that were dominant in the discrimination of the Ova group were IL-1β, IL-13, IL-4, and RANTES, as determined by the first function, whereas the cytokines that were dominant in discriminating the Bud/Ova group from the PBS group comprised IL-9, eotaxin, IL-6, and IL-4.

**DISCUSSION**

Repeated allergen challenges in the sensitized BN rat caused AHR, ASM growth, increased total airway wall area, and goblet cell hyperplasia. There were no significant changes in airway wall thickening on the luminal side of the ASM bundles. Changes in the responsiveness of the lung periphery accounted for AHR, whereas ASM growth affected both central and peripheral airways. The increase in AHR after multiple challenges was not significantly attenuated by Bud (0.1 mg/kg), despite its efficacy against a single challenge. Bud did, however, inhibit the increase in ASM in both central and peripheral airways, prevent the increase in goblet cells in central airways, and inhibit the increased total airway wall area in the peripheral airways. The ASM and goblet cell changes were still present 1 wk after cessation of challenges despite resolution of AHR. These observations indicate that the cause of AHR cannot simply be an increase in ASM mass.

We used both the standard single-compartment model and the constant-phase model of respiratory mechanics to examine the contribution of different lung compartments to responses to inhaled MCh and the effects of allergen exposures on these responses. Measurement of resistance and elastance with the single-compartment model has been most frequently used to assess AHR. However, the constant-phase model provides additional information on the site of pulmonary responses and is increasing in popularity for this reason. The constant-phase model was first applied in dogs by Hantos et al. (15) but has since been used in other species including the rat (1, 24). This model allows the partitioning of responses to large airways...

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based on changes in $R_N$ or to the peripheral lung compartment from changes in $G$ and $H$. The interpretation of changes in $G$ and $H$ is more complex than $R_N$ but reflects a variety of factors, including altered rheological properties of the tissues that may be associated with hyperinflation, for example, and with losses of communicating air spaces because of airway closure (2). $G$ and $H$ will increase proportionately if a fraction of the lung ceases to participate in ventilation through either airway closure or alveolar collapse (2). On the other hand, $G$ will increase more than $H$ if the lung becomes heterogeneous.

The AHR present after a single challenge in our data was detected by the single-compartment model. The changes were detected in $E_{rs}$, indicative of an alteration in the properties of the peripheral lung, and consistent with this interpretation there was also a significant change in the $H$ parameter of the constant-phase model. AHR after three Ova challenges was also associated with a predominantly peripheral lung response but was detected from the $G$ parameter only, implying a difference in the site or mechanisms of AHR resulting from single and multiple challenges. The relatively greater change in $G$ than $H$ suggests that heterogeneous airway narrowing was a substantial contributor to the mechanical response. In no situation was AHR detected by $R_N$, suggesting that the large airways do not contribute to this phenomenon. A limitation of the interpretation of the constant-phase model is that the actual airway sizes or generations corresponding to the $R_N$ and $G$...
parameters have never been confirmed by direct techniques. Our assumption that airways larger than 1 mm in perimeter should be reflected in $R_N$ whereas airways smaller than 1 mm should be captured by $G$ has not been directly verified. However, this cutoff was determined a priori, based on previous classifications of airway size and on knowledge of the distribution of sizes in the rat (29, 30).

A patent difference in single and multiple challenges is that the latter has an association with airway remodeling. ASM mass was increased to similar extents in central and peripheral airways by the Ova challenges, whereas goblet cells were increased to a greater extent in central airways. Increased total airway wall area also affected both central and peripheral airways. The increase in total airway wall area exceeds by far the increase in ASM area and is therefore explained by other components, such as deposition of matrix proteins (29). Despite the increase in ASM in large airways and the increased total large airway wall area, there was no alteration in the responsiveness of these airways as reflected in the $R_N$ parameter of the constant-phase model. An increase in ASM in the large airways has been described in fatal asthma and in severe nonfatal asthma sufferers (3, 6, 8, 28) and has been imputed to be a significant contributor to disease severity. However, it is apparent from the present study that the presence of an increase in ASM mass may not necessarily lead to AHR. Indeed, the lack of association between the remodeling and AHR in the present study is reinforced by the persistence of ASM remodeling at 1 wk after the three Ova challenges, when AHR was no longer present. Recent studies have shown changes in ASM phenotype in similar animal models (26), suggesting the possibility that remodeled muscle may have reduced contractility. Some of these changes may be long-lasting; persistence of ASM remodeling at 35 days after multiple Ova challenges of the rat has been described at a time when AHR was no longer present, supporting our conclusions (23). In contrast, ASM remodeling and AHR both persist after multiple Ova challenges in the murine model (37), but the present data would suggest that the two are not necessarily causally related.

Bud prevented the ASM growth in all sized airways and inhibited goblet cell proliferation in the large airways. These findings suggest that ASM remodeling is relatively sensitive to steroid treatment. The sensitivity of ASM remodeling to corticosteroid treatment may reflect accompanying matrix remodeling, which also occurs in the BN rat after allergen challenges (29). The inhibitory effects of corticosteroid on ASM proliferation are attenuated in the presence of collagen in human cells (4). However, collagen is deposited outside the ASM layer in the allergen-challenged rat (29) and therefore may not have a substantial influence on the sensitivity of ASM remodeling to steroid treatment. A similar study has demonstrated efficacy of ciclesonide and fluticasone against allergen-induced ASM remodeling in the BN rat (22). Steroid-mediated effects on matrix remodeling have been reported for human asthmatics (4). The increased total airway wall area that was observed in the large airways was unaffected by Bud, but increase in total airway wall area in the peripheral airways was prevented. A dose-dependent effect of fluticasone on the attenuation of the deposition of fibronectin following prolonged repeated challenge of the BN rat has also been reported (41). Interestingly, Pini et al. (29) reported that biglycan, collagen, and decorin were prevalent in medium- to large-sized airways, while only biglycan was present in the peripheral airways. Furthermore, the amount of collagen present in the repeated Ova-challenged rats was far greater in the peripheral airways compared with medium to large airways (29). Thus it is possible that the sensitivities of the matrix deposition to steroid may be dependent on not only the particular proteoglycan present but also the amount of various proteoglycans deposited in airways of different sizes. Perhaps the matrix composition is an important underlying factor in the effectiveness of steroid in reducing total airway wall area in the peripheral airways. As there were no differences in airway wall thickening on the luminal side of the smooth muscle bundles in the repeated Ova-challenged animals, this potential aspect of remodeling cannot have contributed to AHR.

Corticosteroid treatment was used to further probe the relationship between AHR and airway remodeling. After a single challenge the increased AHR in response to Ova was inhibited by Bud, but AHR after multiple challenges was not. The difference in treatment effects is consistent with the results of the constant-phase model, indicating a different spatial distribution of the AHR induced with the two different protocols. Furthermore, the data indicate that the remodeling process is an epiphenomenon and is neither necessary nor sufficient for the development of AHR. Given the uncertain relationship between AHR and remodeling, we explored a panel of cytokines and chemokines found in BAL fluid 48 h after three Ova challenges and after Bud. We used LDA to examine the cytokines/chemokines tested as discriminating factors into the three treatments: PBS, Ova, and Bud+Ova. Kaminska et al. (18) performed a similar analysis, the principal component analysis, to determine dominant mediators from the sputum in asthmatic individuals with fixed airflow obstruction in whom remodeling was particularly marked. In their study, the key variables were IL-12, IL-13, and IFN-γ in the chronically obstructed group and IL-9, IL-17, monocyte chemotactic protein 1 (MCP-1), and RANTES in the intermittently obstructed group (18). Interestingly, in our study, the principal discriminants of the repeated Ova-challenged group were IL-1β, IL-4, IL-13, and RANTES. IL-4 and IL-13 are well-established mediators of Th2 inflammation in allergic asthma (16, 45). IL-1β is a proinflammatory cytokine that is reportedly upregulated in BALs of corticosteroid-resistant patients (14). RANTES is a chemokine for memory T lymphocytes and monocytes and has been demonstrated to increase in the BAL of asthmatic patients (25). IL-9 may lead to upregulation of mucus production (40) and is also associated with the upregulation of the antiapoptotic molecule Bcl-2 (35). Moreover, corticosteroids have been implicated in the apoptosis of various immune cell types and epithelial cells (9, 10). Thus IL-9 along with the associated Bcl-2 could perhaps be protective against cell apoptosis induced by corticosteroids and thus play a role in discriminating animals in the Bud+Ova group.

In conclusion, the data indicate that the site of AHR following allergen exposure is not consistent with the pattern of increased total airway wall area, airway wall thickening, remodeling of ASM, and goblet cell hyperplasia. Likewise, the changes in AHR were more resistant to steroid therapy than ASM and goblet cell remodeling. Loss of contractile phenotype, which has been described for ASM in culture and in vivo after repeated allergen challenge in the rat, could account for a lack of association between ASM mass and AHR, but whether
it accounts for our findings requires investigation. The cytokine/chemokine data suggest some of the important mediators that are associated with allergic inflammation and may account for AHR independently of ASM remodeling, but their relevance for airway remodeling in asthma requires further exploration.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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