

Heaves, an asthma-like equine disease, involves airway smooth muscle remodeling

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Background: Increased airway smooth muscle mass is a prominent feature of asthmatic airway remodeling. Airway smooth muscle hyperplasia occurs in rodent models of experimental asthma, but the relevance of such finding to spontaneously occurring disease in large mammals is unknown. **Objective:** We examined horses with heaves, a naturally occurring equine asthma related to sensitization and exposure to moldy hay. We hypothesized that airway remodeling occurs in heaves and shares disease mechanisms with asthma.

Methods: We quantified the airway smooth muscle mass and the numbers of proliferating and apoptotic airway smooth muscle cells in 5 horses with heaves and 5 control horses using morphometric techniques. Cell proliferation was detected in tissue sections by immunostaining for proliferating cell nuclear antigen, and apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling of fragmented DNA. Both signals were colocalized with smooth muscle specific α -actin.

Results: Horses with heaves had a significant increase in the amount of smooth muscle in the airways (nearly triple that of the controls) associated with increased myocyte proliferation (7-fold proliferating cell nuclear antigen-positive airway myocytes) and apoptosis (6-fold).

Conclusion: Heaves involves airway smooth muscle growth associated with myocyte hyperplasia, which may contribute to the growth, and increased myocyte apoptosis that may reflect a compensatory mechanism serving to limit the abnormal smooth muscle growth.

Clinical implications: Airway smooth muscle remodeling in heaves may be involved in the mechanism of airway

hyperresponsiveness and chronic lung function impairment in a way comparable to human asthma. (*J Allergy Clin Immunol* ■■■■;■■■;■■■-■■■.)

Key words: Airway obstruction, apoptosis, animal, disease models, hyperplasia, smooth muscle cells

Asthma is a chronic inflammatory condition associated with airway remodeling,¹ a complex series of structural changes in the airways, which includes increases in the amount of airway smooth muscle (ASM) corrected by airway size (ASM mass). The increase in ASM mass is likely important in the causation of airways hyperresponsiveness, as suggested by mathematical modeling of airway mechanics²⁻⁴ and experimental data demonstrating a correlation between smooth muscle growth and airway hyperresponsiveness.⁵ Data from the Brown Norway (BN) rat model of experimental asthma also suggest that the increase in ASM mass is caused at least in part by myocyte hyperplasia.⁶

Although rat and mouse models have contributed valuable data on biological mechanisms of ASM growth that may operate in human asthma, the relevance of rodent-based experimental models to human airway remodeling may be influenced by factors related to animal size and cell turnover. Rodent models of induced disease may also have limitations in representing the pathogenesis of actual asthma as a naturally occurring, chronic disease. Here we hypothesized that heaves, a spontaneously occurring equine asthma-like disease, involves ASM remodeling. Heaves is an obstructive pulmonary disease that develops in horses exposed to moldy hay, characterized by chronic airway inflammation, airway hyperresponsiveness, and episodes of bronchoconstriction that may improve in response to β_2 -adrenergic agonists and corticosteroids.⁷⁻⁹ The respiratory symptoms are associated with elevated levels of IgE in bronchoalveolar lavage^{10,11} and serum,¹² supporting the role of type I hypersensitivity reactions to inhaled molds and fungi in dusty hay. Early removal from hay exposure may be accompanied by remission of symptoms, whereas continued exposure may lead to irreversible airflow limitation. Thus, heaves, similarly to asthma in human beings, may involve remodeling of the airway wall as part of its pathogenesis. Horses with heaves may therefore be a suitable animal model of asthmatic airway remodeling, having the characteristics of a chronic and spontaneously occurring disease. In this study, we analyzed the smooth muscle mass and the frequency of

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Abbreviations used

α -SMA: Smooth muscle α -actin
 ASM: Airway smooth muscle
 BN: Brown Norway
 PCNA: Proliferating cell-associated nuclear antigen
 TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

myocyte proliferation and apoptosis in airway specimens obtained from horses with heaves. We found a significantly increased ASM mass compared with controls, as well as evidence of hyperplasia and increased apoptosis of ASM cells.

METHODS

Lung specimens

Lung tissue blocks sampling airways of different sizes were harvested at autopsy from 5 horses with a diagnosis of heaves based on a history of chronic respiratory disease, compatible clinical manifestations, and abnormal lung function. These horses were kept in pasture during the summer months and were stabled during the winter, and had an increase in neutrophils (>5%) in bronchoalveolar lavage fluid when stabled and fed hay. Lung tissues from 5 adult control horses were obtained from a local slaughterhouse. The control horses had no clinical evidence of respiratory disease, and the absence of obvious respiratory illness was confirmed by gross pathology. Both heaves-affected and control horses were older than 10 years (range, 12–21 years) with the exception of 1 control horse (3 years), and both groups were made up of male and female horses. The tissue specimens were formalin-fixed and paraffin-embedded.

Detection of ASM cell proliferation and apoptosis, and signal colocalization

Immunohistochemistry was performed for the colocalization of proliferating cell-associated nuclear antigen (PCNA) with smooth muscle α -actin (α -SMA). The following mAbs were used: clone 1A4 to α -SMA (Sigma-Aldrich, St Louis, Mo) and clone Ab-1 to PCNA (Calbiochem, San Diego, Calif). The tissue sections underwent a high-temperature epitope unmasking treatment in antigen retrieval buffer (Vector Laboratories, Burlingame, Calif) at 95°C for 30 minutes. Cell membranes were permeabilized in 0.2% Triton X-100 (Sigma-Aldrich) in pH 7.6 Trizma buffer. The tissue sections were blocked in universal blocking solution (Dako, Carpinteria, Calif) supplemented with 20% normal goat serum (Vector Laboratories) and incubated with anti-PCNA antibody at 37°C for 1 hour. On negative control sections, the primary antibody was replaced by a mouse isotype IgG control at the same concentration. The detection system consisted of a biotinylated goat antimouse IgG polyclonal antibody (BD Biosciences, Mississauga, Québec, Canada), an avidin/biotin-alkaline phosphatase complex (Vector Laboratories), and a BCIP/NTB chromogen substrate (Vector Laboratories), which produces a violet precipitate. The PCNA immunostaining was followed by immunostaining for α -SMA using the same avidin/biotin-alkaline phosphatase complex detection method with Vector-Red chromogen substrate (Vector Laboratories) as a developer. The sections were then counterstained with methyl green, dehydrated, and mounted.

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling of fragmented DNA (TUNEL; Apop-Tag kit; Qbiogene Inc, Carlsbad, Calif) as per the manufacturer's instructions, and the signal was developed with

diaminobenzidine-nickel (Vector Laboratories). Colocalization with α -SMA followed as for PCNA.

Quantitative morphology

Morphometry was performed on cross-sections of complete bronchioles and small to medium bronchi, and arc sectors of larger bronchi. Each airway was mapped with partially overlapping microscopic fields recorded as calibrated digital image files, and the complete airway was then reconstructed as a single large image using commercial software (Adobe Photoshop; Adobe Systems Inc, San José, Calif). α -Actin immunostained ASM bundles were subtracted from the rest of the tissue components, and the total ASM surface area and length of the airway basement membrane were measured by using Image-Pro Plus analysis software (MediaCybernetics, Carlsbad, Calif). To standardize for airway size, the ASM surface area and the counts of PCNA⁺ and TUNEL⁺ cells were divided by the basement membrane perimeter squared (P_{BM}^2). The basement membrane perimeter is a constant dimension in an airway section, regardless of the degree of ASM contraction and lung inflation.¹³ For arc sectors of airways larger than the tissue blocks, an estimate of airway size was geometrically determined by fitting the airway arc sector into an ellipse and applying the following formula:

$$P_{BM} = \frac{ARC_{BM} \cdot E}{ARC_E}$$

where P_{BM} is the estimated basement membrane perimeter of the complete airway section, ARC_{BM} is the measured length of the irregularly shaped basement membrane contained in the airway sample arc sector, ARC_E is the length of the perfect elliptic arc that fits the airway arc, and E is the perimeter of the complete ellipse.

Statistical analysis

Data are expressed as means $\pm$ SEMs. PCNA⁺ and TUNEL⁺ cell counts are expressed as cells/mm². ASM mass is a dimensionless index measured as ASM surface area divided by P_{BM}^2 . Measurements were performed in 6.4 ± 0.7 airways/horse (range, 4–10) made up of different airway sizes. Group comparisons were analyzed with the Student *t* test. Associations were analyzed by least-square linear regression or best fit nonlinear regression and the coefficient of correlation (*R*) or the coefficient of determination (*R*²). *P* values less than .05 were considered significant. Data analysis was performed with SPSS (SPSS Inc, Chicago, Ill) and SigmaPlot (Systat Software Inc, Richmond, Calif).

RESULTS

ASM mass

The lung sections of horses with heaves showed evidence of increased mucus production, epithelial damage, plugging of the airway lumen with mucous material and epithelial debris, presence of inflammatory cell infiltrates in the airway wall and in a peribronchial location, and an overall airway wall thickening affecting all tissue layers (Fig 1, A and B). To demonstrate airway remodeling, we quantitatively compared the ASM mass. The horses with heaves had a 2.8-fold increase compared with the control horses ($9.15 \pm 1.38 \cdot 10^{-3}$ vs $3.21 \pm 0.23 \cdot 10^{-3}$, respectively; *P* = .003; Fig 1, C).

ASM cell proliferation and apoptosis

To test the hypothesis that myocyte hyperplasia may contribute to ASM growth in heaves, we colocalized PCNA immunodetection with α -SMA (Fig 2, A and B)

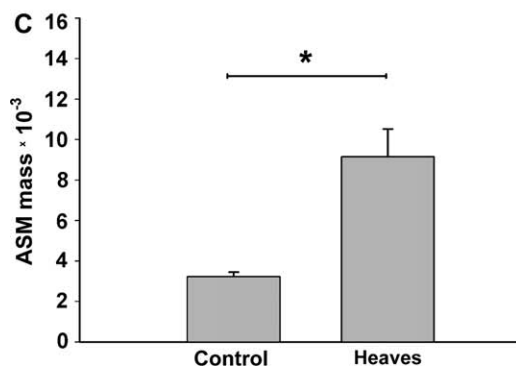
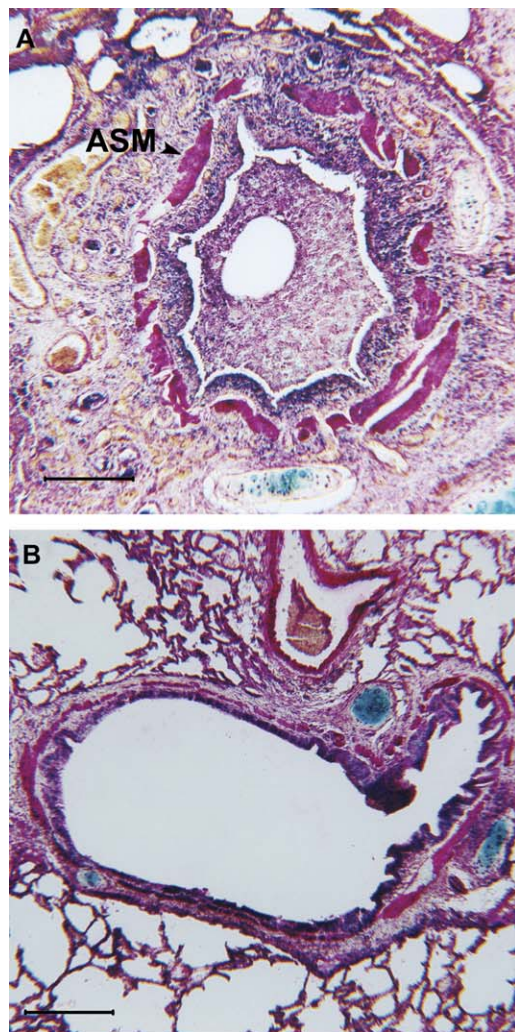


FIG 1. ASM growth in heaves. **A**, Small bronchus of a horse with heaves. **B**, Size-matched bronchus in a control horse. **C**, ASM mass, heaves vs control groups. Scale bars: 250 μ m. * $P < .05$.

and measured the numbers of PCNA⁺ smooth muscle cells corrected by P_{BM}^2 . The PCNA⁺ cells/mm² were increased in heaves by 7.2-fold compared with the control horses (6.41 ± 1.26 vs 0.89 ± 0.27 cells/mm², respectively; $P = .003$; Fig 2, C).

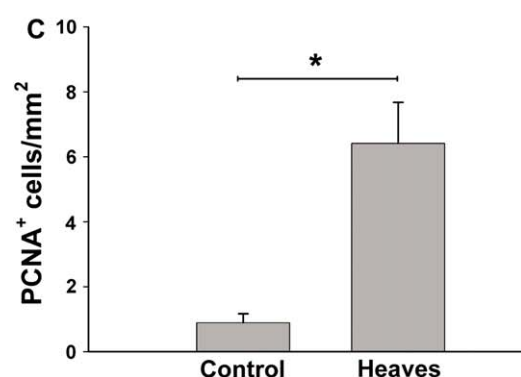
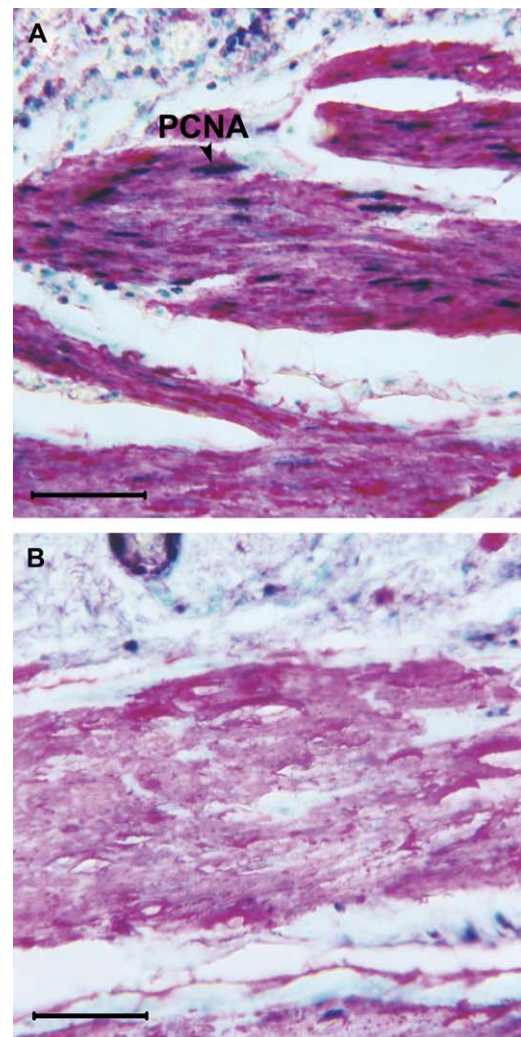


FIG 2. Immunostaining for PCNA (nuclear dark signal) colocalized with α -SMA (cytoplasmic red signal). **A**, High-magnification detail. **B**, Equivalent microscopic field in a size-matched airway of a control horse. **C**, PCNA⁺ cell numbers/mm². Scale bars: 50 μ m. * $P < .05$.

Because apoptosis may participate in smooth muscle cell turnover and the maintenance of tissue homeostasis, we tested the hypothesis that an alteration in the rate of myocyte apoptosis may participate in the abnormal

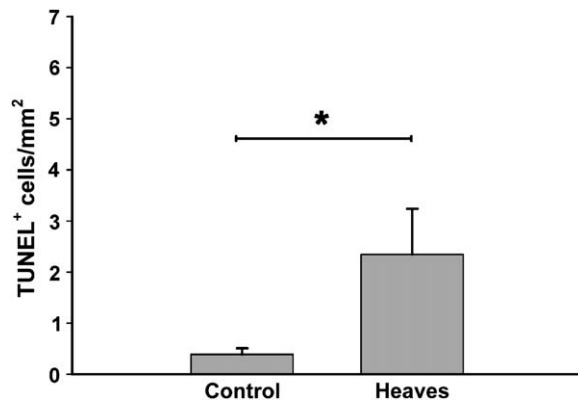


FIG 3. Apoptosis detected by TUNEL and colocalized with α -SMA. The plot represents TUNEL⁺ cells/mm² in heaves vs controls. * $P < .05$.

growth of ASM detected in heaves. Using an approach similar to PCNA, the TUNEL⁺ nuclear signal of apoptotic cells was colocalized with α -SMA, and the frequency of apoptotic ASM cells was measured. There was a 6-fold increase in the TUNEL⁺ cells/mm² in the horses with heaves compared with the controls (2.34 ± 0.90 vs 0.39 ± 0.12 ; $P = .0004$; Fig 3).

Effect of airway size on remodeling changes

In the control horses, the ASM mass and the normalized numbers of PCNA⁺ and TUNEL⁺ cells/mm² were approximately constant at the different airway sizes analyzed, resulting in an almost flat linear regression function (slope = -0.04 , -0.05 , and -0.02 for the ASM mass, PCNA⁺, and TUNEL⁺ cells/mm², respectively; Fig 4). This is consistent with using P_{BM}^2 for data normalization as an appropriate standardization for airway size.

Consistently with the differences observed between the heaves and control groups, the respective regression functions that relate ASM mass and the numbers of proliferating and apoptotic cells with airway size were displaced upward to higher ordinate values in the heaves group (Fig 4). In the presence of heaves, the airway size influenced the increases in ASM mass ($R = -0.68$; $P = .001$) and PCNA⁺ cells/mm² ($R = -0.45$; $P = .002$); the increments were larger the smaller the airways. These data suggest that heaves involves hyperplastic smooth muscle growth in all airway sizes, but the effect is more marked in small airways. It is unclear whether this is also the case for the increase in TUNEL⁺ cells/mm², because the data are inconclusive about the influence of airway size ($P = .656$; power = 14.9%; Fig 4, C).

Relationship between proliferation and apoptosis

The ASM mass correlated with the numbers of PCNA⁺ cells/mm² ($R = 0.57$; $P < .0001$; Fig 5, A), supporting the idea that myocyte hyperplasia contributes to the growth of ASM. A weak positive association was found between the numbers of proliferating and apoptotic smooth muscle cells ($R = 0.34$; $P = .042$; Fig 5, B). This result was

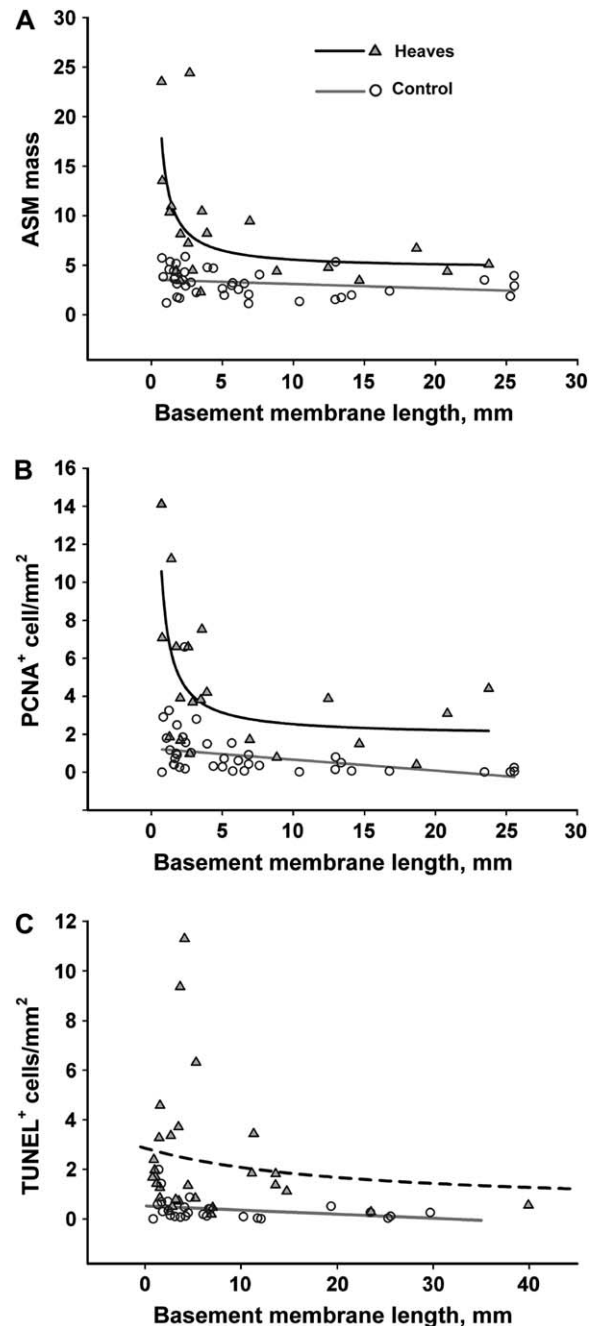


FIG 4. Regression analysis of the ASM mass (A), and the numbers of PCNA⁺ (B) and TUNEL⁺ (C) ASM cells versus basement membrane length. Control horses: circles and flat (thin) linear regression functions. Heaves: triangles and thick regression curves. In heaves, the increments in ASM mass (A) and proliferating cells (B) are larger the smaller the airways.

inferred by correlating the numbers of PCNA⁺ and TUNEL⁺ cells/mm² in airways matched by size (<10% difference in basement membrane length) in each study group separately. This approach is limited by the fact that PCNA immunostaining and TUNEL were performed on different preparations, and only some airway sections

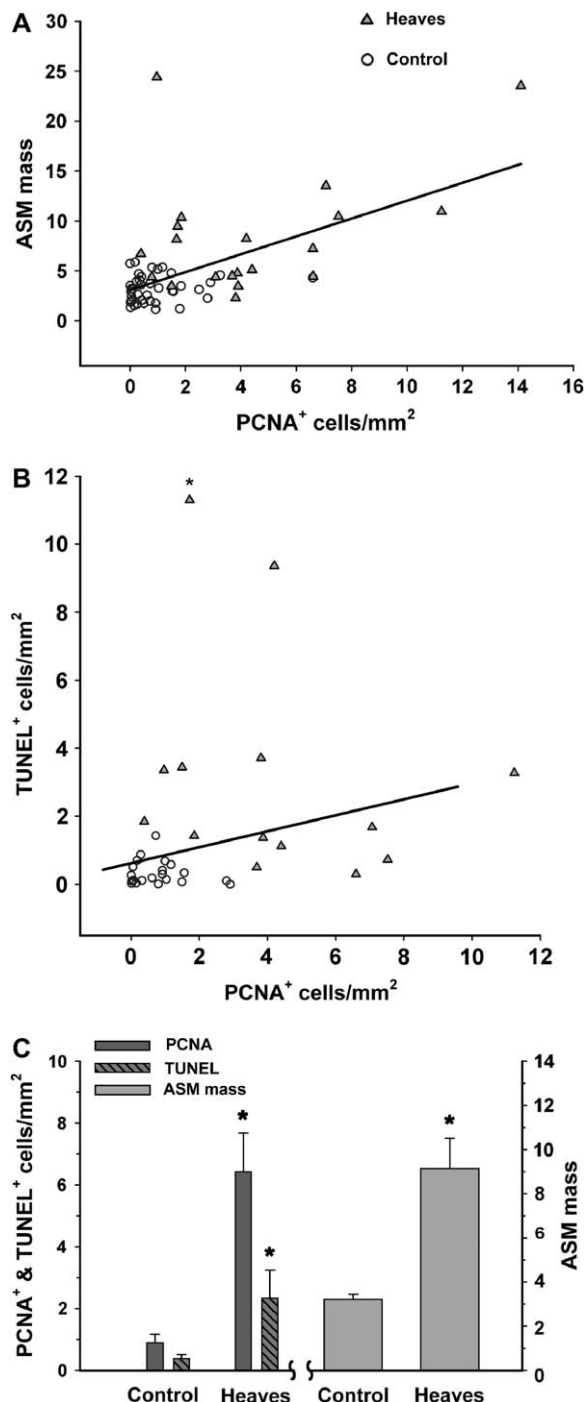


FIG 5. Regression analysis of (A) ASM mass and (B) apoptotic cells versus proliferating ASM cells/mm². *Outlier. C, Summary graph of the increases in proliferating (7-fold) and apoptotic (6-fold) myocytes and ASM mass (close to 3-fold). * $P < .05$ vs control.

($n = 36$) met the inclusion criteria for this analysis, resulting in a low statistical power (53%). Thus, the weakness of the association detected does not rule out the possibility of a stronger relationship between smooth muscle cell proliferation and apoptosis. Overall, the data reflect a

concomitant increase in the numbers of proliferating and apoptotic ASM cells in heaves (Fig 5, C), suggesting that the increase in the frequency of apoptotic myocytes may develop in the course of remodeling as a compensatory mechanism to limit the increase in ASM mass.

DISCUSSION

Human asthma involves a series of structural changes of the airways that occur in association with chronic airway inflammation and may result from sustained and abnormal repair responses. Such changes, termed *airway remodeling*, have recently received considerable attention because they likely underlie the pathogenic mechanisms leading to airway hyperresponsiveness, abnormal airway narrowing, and, eventually, irreversibly impaired lung function.^{14,15} Airway remodeling may occur from the early stages of the disease, perhaps antedating symptoms. The extent of the structural alterations may be closely related to the disease severity and largely account for the cases of difficult asthma. Airway remodeling involves alterations such as goblet cell and mucous gland hyperplasia, subepithelial fibrosis, neovascularization, ASM growth, and an overall thickening of the airway wall.¹ The increase in ASM mass is a particularly significant change that has been observed in human beings with asthma¹⁶⁻²⁰ and experimentally reproduced in rodent models of allergic sensitization and repeated airway challenge.^{5,21-25} In the BN rat, the *in vivo* modeling of ASM growth suggests that myocyte hyperplasia contributes at least in part to the increase in ASM mass.⁶ Mathematical modeling of airway mechanics,²⁻⁴ and experimental data correlating the ASM mass with the responsiveness to challenge with cholinergic agonists,⁵ suggest that the growth of ASM is sufficient to explain airway hyperresponsiveness. Although the ASM may be intrinsically normal in asthma, an increased ASM mass may increase airway narrowing through an augmentation in force generation and by an amplification of the effect of smooth muscle fiber shortening.²⁻⁴

Despite the ability to reproduce ASM remodeling in experimental asthma, the models used, based almost exclusively on rodents, would benefit from further assessment of the extent to which their features can be translated to human asthma. Two issues need to be considered in this regard. One is the possible influence of animal size on the dynamics of cell turnover and its potential relevance to the roles of proliferation and apoptosis. The other is the ability of rodent models, where induced sensitization is followed by repeated airway challenge within a relatively short period, to represent the chronicity of naturally occurring disease, and the long-term alterations associated with it. In this regard, heaves is a spontaneously occurring disease that affects a large mammal and closely parallels human asthma.^{7,26} Tools such as bronchial biopsy, percutaneous lung biopsy, or a thoracoscopically guided wedge pulmonary resection are feasible and can be used safely in horses.^{27,28} Moreover, the prospective study of horse cohorts with controlled exposure to moldy hay in an

experimental setting may allow the analysis of disease mechanisms in early asymptomatic stages, an approach not feasible in human beings. The airways of horses with heaves show many of the pathophysiological features of extrinsic human asthma. There is chronic airway inflammation, episodes of airway obstruction induced by antigen inhalation (moldy hay), and airway hyperresponsiveness to histamine and methacholine.⁷ Similar to asthma, the symptoms are relieved by β_2 -adrenergic agonists and corticosteroids.²⁹ The airway inflammatory infiltrates are associated with a T_H2 activation phenotype,³⁰ and the affected horses have a positive passive cutaneous anaphylaxis test³¹ and elevated levels of IgE in bronchoalveolar lavage^{10,11} and serum.¹² A difference with human asthma is that the infiltrating granulocytes are predominantly neutrophils,^{7,30} although eosinophils are also present in the airway tissues. Heaves is triggered by moldy hay, but it is not a form of extrinsic allergic alveolitis.

In the current study, our data show for the first time that heaves also parallels human asthma in terms of the presence of airway remodeling involving the smooth muscle, and suggest that similar mechanisms of ASM growth may be shared. Compared with disease-free horses, the horses with heaves had a significant increase in ASM mass associated with evidence of increased myocyte proliferation. Furthermore, a correlation between the ASM mass and the frequency of PCNA⁺ ASM cells was found. These data suggest that myocyte hyperplasia may contribute to the ASM growth in heaves. In human asthma, the growth of ASM has been attributed to both hyperplasia and cell hypertrophy,^{17,32,33} although some uncertainty remains regarding the ability to detect cell proliferation markers (proteins expressed during the S phase of the cell cycle) by immunohistochemistry in bronchial biopsies of patients with asthma.³⁴ Compared with the analysis of whole airway wall sections, the detection of proliferation markers in superficial bronchial biopsies may be less than ideal to capture the positive ASM cells, which may be relatively low-frequency events. ASM cells obtained from bronchial biopsies of patients with asthma, however, showed higher proliferation rates when expanded in culture,³⁵ consistent with earlier reports of an association between airway hyperresponsiveness and increased sensitivity of ASM to growth factors.³⁶

Our data show an increase in the rate of apoptotic ASM cells in heaves. The role of apoptosis in the physiological turnover of smooth muscle cells has not been defined to date. Some studies have suggested that receptor-mediated apoptosis may play a role in the control of myocyte numbers because both airway and vascular smooth muscle cells constitutively express Fas and undergo apoptosis on Fas ligation *in vitro*.^{37,38} In atherosclerosis, the progression of atheromatous plaques involves T-cell inflammatory infiltrates in the arterial wall and growth of the vascular smooth muscle associated with an increase in myocyte apoptosis.³⁷ Our data showing an increase in ASM mass associated with increases in both myocyte proliferation and apoptosis in heaves suggest a role for increased myocyte apoptosis as a compensatory mechanism for the hyperplastic growth of ASM.

The influence of airway size in remodeling is unclear, and data from separate studies differ. Here we observed an increase in ASM mass and PCNA⁺ and TUNEL⁺ myocytes at all airway sizes, although the increments in ASM mass and PCNA⁺ cells were progressively greater the smaller the airways. In the BN rat, the growth of ASM was greater in larger airways in actively sensitized animals,⁵ whereas an adoptive transfer T-cell-driven model showed an influence of airway size similar to the pattern detected here in horses.³⁹ In human asthma, the pattern of anatomic distribution of ASM growth, and the relative contribution of myocyte hyperplasia and hypertrophy to ASM accumulation at different airway sizes, remain controversial.³³ The variety of patterns seen in different studies and models may result from varying observational conditions and techniques, and may also reflect varying factors such as the sites of preferential deposition of allergen, the function of antigen presenting cells, and the local inflammatory activity.

In summary, heaves, an asthma-like equine disease, involves airway remodeling. As in human beings with asthma and rodent models of experimental asthma, there is an increase in the ASM mass that may underlie the mechanism of airway hyperresponsiveness and airflow obstruction in the affected horses. Myocyte hyperplasia may cause at least in part the abnormal growth of ASM, and a concomitant increase in apoptosis may play a compensatory role. Because heaves is a naturally occurring condition, it may be a suitable model of spontaneous disease onset, chronicity, and ASM remodeling in a large mammal. This model creates an opportunity for studies aimed at filling current gaps in our knowledge such as the time course of remodeling during early disease, when the initiation of structural changes in the airways may antedate symptoms or detectable changes in lung function. The conclusions from such studies may be useful in the testing of early therapeutic interventions of relevance to human asthma.

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