Role of cellular effectors in the emergence of ventilation defects during allergic bronchoconstriction

Skander Layachi,¹ Liisa Porra,²,³ Gergely Albu,⁴ Nathalie Trouillet,⁵ Heikki Suhonen,³ Ferenc Peták,⁶ Henri Sevestre,⁵ Pekka Suortti,² Anssi Sovijärvi,⁷ Walid Habre,⁴ and Sam Bayat¹

¹Université de Picardie Jules Verne and Amiens University Hospital, Amiens, France; ²Department of Physics, University of Helsinki, Helsinki, Finland; ³European Synchrotron Radiation Facility, Grenoble, France; ⁴Geneva Children’s Hospital, University Hospitals of Geneva, and Geneva University, Geneva, Switzerland; ⁵Department of Pathology, Amiens University Hospital, Amiens, France; ⁶Department of Medical Physics and Informatics, University of Szeged, Szeged, Hungary; and ⁷Department of Clinical Physiology and Nuclear Medicine, Helsinki University Central Hospital, Helsinki, Finland

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Recent computational modeling studies suggest that homogeneous airway constriction can appear even in the absence of heterogeneous smooth muscle activation. Venegas and colleagues in an integrative computational model of the bronchial tree demonstrated that due to feedback between regional tidal expansion and airway constriction, even a uniform activation of airway smooth muscle above a critical level led to the self-organized emergence of patchy areas of ventilation defects (VDs) resulting from clustered peripheral airway narrowing and closures (37). However, it is not known whether local factors within the airway wall or parenchyma may influence the emergence and spatial distribution of VDs, thereby modulating the dynamic system behavior of the lung during bronchoconstriction.

Asthma is characterized by chronic inflammation of the airways. Allergen challenge in sensitized subjects causes transient increases in the number of total cells, eosinophils, T cells, mast cells, and neutrophils in bronchoalveolar lavage (BAL) fluid (25) and inflammatory cell infiltration of airway walls as revealed by bronchial biopsies (11). In the Brown Norway rat, a strain genetically predisposed to allergic hypersensitivity (19), allergen provocation following active sensitization produces patchy inflammatory cellular infiltrates in the lung parenchyma, and in the walls of both airways and blood vessels (8). Cellular effectors can contribute to airway hyperresponsiveness through the expression of inflammatory mediators that include cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors (2). Eosinophils are believed to play a central role in allergic inflammation in atopic asthma. However, the relation between eosinophils, airway responsiveness and lung function remains poorly understood.

The present study was undertaken to assess the relation between the spatial distributions of cellular effectors and the emergence of defects in regional ventilation distribution following allergen challenge. For this purpose, a high spatial resolution synchrotron imaging technique was developed to study the emergence of VDs following allergen provocation in ovalbumin (OVA)-sensitized Brown Norway rats. To better separate airway and respiratory tissue mechanical changes following acute allergen challenge, forced oscillation respiratory impedance measurements were used in combination with synchrotron imaging.

METHODS

Animal preparation. Animal care and experimental procedures were in accordance with the Guide for the Care and Use of Labora-
The diameters (Daw) of the two largest central airways in each lung image were calculated by fitting an ellipse to the lumen in tissue-density CT images. Because airways were not always perpendicular to the image plane, the small axis of the ellipse was measured as the inner radius of the airway in the middle and caudal slices (5). The cumulated central airway diameter (Daw cum) was calculated as the sum of the Daws of the two largest central airways in each lung image.

Measurement of respiratory mechanics. At end-expiratory, the mechanical ventilation was paused, a loudspeaker-in-box system and the tracheal cannula were connected through a polyethylene tube (100 cm length, 2.0 mm ID), and a small-amplitude (1 cmH2O trough to peak) forcing signal was delivered into the trachea. All measurements were performed at zero end-expiratory pressure. The loudspeaker was driven by a computer-generated pseudorandom signal ranging from 0.5 to 21 Hz. Lateral pressures were measured at the loudspeaker end (P1) and the distal end (P2) of the wave-tube with miniature sidearm transducers (ICS 33NA00D). These pressure signals were low-pass-filtered (<25 Hz) and digitized at a sampling frequency of 128 Hz. The inverse transfer function (P1/P2) was created by fast Fourier transformation from the 8-s recording. The input impedance of the respiratory system (Zrs) was computed from the pressure transfer function as the load impedance of the wave tube (28) by using the transmission line theory (36):

\[
Z_{rs} = Z_0 + \sinh(\gamma L)/(\{P_1/P_2 - \cosh(\gamma L)\})
\]

where \(Z_0\) is the characteristic impedance of the wave tube and \(\gamma\) is the complex propagation wave number; these were determined from the geometrical parameters of the wave tube, and the material constants of the tube and the gas in it. Three to five Zrs spectra were ensemble-averaged under each experimental condition.

A model that includes Newtonian resistance (Rn), inertances (Iaw) in series with constant-phase tissue compartments incorporating tissue damping (G) and elastance (H) was fitted to the averaged Zrs data (15). The data at frequencies coinciding with the heart rate and its harmonics were often corrupted, as evidenced by poor coherence and a high standard deviation (SD), and they were omitted from the model fitting.

Study protocol. Each animal served as its own control. Approximately 10 min was allowed for each protocol to verify stable condition prior to baseline data acquisitions. On the basis of a reference anteroposterior projection image, two different axial image slice levels were selected approximately at the sixth (middle) and eighth (caudal) thoracic vertebral levels for tomographic imaging sequences. The volume history was standardized by inflating the lungs to the total lung capacity (30 cmH2O). Three to four Zrs recordings were then collected during end-expiratory pauses of the mechanical ventilation (Fig. 1). These were completed by KES subtraction image acquisition following 18 tidal inhalations of a 70% Xe-30% O2 gas mixture. The number of respiratory cycles preceding the image acquisition was based on preliminary experiments assessing xenon wash-in vs. the number of xenon inhalations. Following baseline data acquisition, the animals were challenged with inhaled 5% OVA using an ultrasonic nebulizer (SAM LS2000; Villeneuve sur Lot, France), after which data acquisition was repeated every 4 min, up to 30 min.

Histological analysis. The animals were euthanized by sodium thiopental overdose, and the lungs were removed and fixed by slow in situ inflation to 25 cmH2O with 10% formalin. The lungs were included in gelatin, cut in transverse sections, and embedded in paraffin. Serial 4- to 5-μm adjacent slices with the best anatomic match to the CT image were cut, deposited on a single slide, and stained with (1) hematoxylin and eosin; (2) Modified May-Grünwald Giems (MGG); and (3) for immunohistochemical (IHC) staining, with monoclonal anti-rat CD68 antibody (AbD Serotec, Colmar, France), because a preliminary study revealed that a substantial part of the cellular infiltrate in this model consisted of macrophages. Slides were acquired and subtracted during inhalation of the Xe-O2 gas mixture. Two CT images are thus simultaneously acquired and subtracted during inhalation of the Xe-O2 gas mixture. Two CT images are thus simultaneously 

instrumental setup has been extensively discussed in previous studies (30, 31). This xenon-density image allows the selection of monochromatic beams from the full X-ray source are required because as opposed to standard X-ray sources, they allow the selection of monochromatic beams from the full X-ray spectrum while conserving enough intensity for imaging with sufficient temporal resolution. In the present study, we used a Fast-Readout Low Noise detector (FRELON; European Synchrotron Radiation Facility, Grenoble, France) with a pixel size of 47 μm. For each CT image, 1,800 projections were obtained in 10 s during end-expiratory apnea, following 18 tidal inhalations of a 70% Xe-30% O2 gas mixture.

Image analysis. Images were processed using the MatLab programming package (Mathworks, Natick, MA). The lung was selected within the monochromatic CT images by segmentation on the basis of tissue density. On the assumption that the constriction or obstruction of airways leading to a lung region leads to a decreased local xenon concentration, the area of well-ventilated lung regions (VA) was defined as areas where the xenon concentration was higher than the median (μ) of the distribution minus two standard deviations: μ-2σ, after a standardized amount of ventilation with the Xe-O2 gas mixture. The remaining lung areas were defined as VD.
digitized using a light microscope coupled to a charged-couple device camera (Zeiss, Feldbach, Germany) and equipped with a motorized tray. Bronchi, blood vessels, and parenchymal regions of interest (ROIs) were randomly selected within or outside of VDs, which were delineated on the basis of xenon-KES CT images. The VD areas were determined by segmentation on the basis of regional xenon concentration as described above. An image mask of the VD was transferred to a low-resolution image of the histology slide with the closest possible anatomic match to the KES CT image following rotation, translation, and rescaling to match the two images. Care was taken to select ROIs that were not in contact with the boundaries of the VD mask. Selected ROIs were then rescanned at high resolution for analysis using the Visilog microscopy software (version 6.8; Noesis, Les Ulis, France). The ROI surfaces and basement membrane (BM) lengths were also determined using the Visilog software. Total cell nuclei irrespective of cell type, and eosinophils were counted in MGG images, both in parenchymal ROIs, expressed as cell density, and within a distance of 100 μm below the bronchial or vascular BMs, expressed as cells per BM length, using computer-assisted image analysis (Visilog 6.8) at ×400 magnification. In each animal, nine blood vessels, nine bronchi, and six parenchymal ROIs were analyzed per stain type and per axial slice level within the VDs and the same number outside. Average data per animal and per topographic location with respect to the VD were considered as the final result. No nonspecific positive staining was observed in the IHC slides. Cells with positive CD68+ immunostaining were counted using automated image analysis (Visilog 6.8) within corresponding ROIs. A single slide was analyzed per stain type and per axial image level.

Statistical analysis. The scatter in the data was expressed as means ± standard error (SE). The Kolmogorov-Smirnov test was used to test data for normality. Depending on the normality of data distribution, a Student’s t-test or Mann-Whitney rank sum test were used to test differences between baseline and postchallenge respiratory mechanics and image-derived data. We used the Student’s unpaired t-test to analyze histological data. Each test was performed with a significance level of \( P < 0.05 \).

RESULTS

Airway and lung peripheral responses to antigen challenge. A representative example of tissue density and xenon distribution images is shown in Fig. 2. Following OVA inhalation challenge, the distribution of xenon became heterogeneous, with emergence of patchy areas with low xenon concentration due to poor regional ventilation. The minimum VA, the maximum coefficient of variation (CV) of regional xenon concentration, and the minimum value of the sum of right and left central airway diameters (Daw) during bronchoconstriction in the 30 min following OVA inhalation, are shown in Fig. 3. The VA decreased \(( P = 0.023)\) following OVA challenge. On the other hand, the CV of xenon concentration increased as ventilation distribution became more heterogeneous \(( P = 0.030)\) following challenge. After the challenge, Daw showed a small but significant decrease \(( P = 0.017)\). Parallel to this decrease, \( R_n \) significantly increased \(( P < 0.001)\) with a large variability between animals. Similarly, tissue damping \(( P = 0.02)\) and elastance \(( P = 0.03)\) increased significantly following allergen challenge. Also, hysteresivity \(( \eta \) calculated as the G/H ratio increased \(( P = 0.002)\).
Histological analysis. Total cell, CD68+, and eosinophil cellular densities in airway and vascular walls as well as in the parenchyma are represented in Fig. 4. Histological analysis was performed randomly in 6 out of 12 protocol animals and in 3 additional nonsensitized control animals. Eosinophil and CD68+ counts were significantly higher in the airway walls (\( P = 0.041 \) and 0.002, respectively), blood vessel walls (\( P = 0.020 \) and 0.015), and in parenchyma (\( P = 0.038 \) and 0.001) inside the VDs compared with their counterparts outside of the VDs. The same observation was made for total cells in the parenchyma (\( P = 0.033 \)) and blood vessel walls (\( P = 0.018 \)). Also, the eosinophil and CD68+ cell densities were significantly higher in both blood vessel walls (\( P = 0.020 \) and 0.034, respectively) and parenchyma (\( P = 0.017 \) and 0.025) within the VDs compared with controls. Total cells within the parenchyma (\( P = 0.037 \)) and vascular walls (\( P = 0.049 \)) were significantly increased compared with control within the poorly ventilated zones.

We found that the minimal Daw during bronchoconstriction following OVA challenge was closely correlated with mean eosinophil and total cell counts in the airway walls within the poorly ventilated zones, as demonstrated in Fig. 5. We did not find a statistically significant correlation between this parameter and CD68+ cells, nor with cell counts within the parenchyma or vascular walls (data not shown).
DISCUSSION

The present study was undertaken to assess the relationship between the distribution of cellular effectors and the emergence of defects in regional ventilation distribution following allergen challenge. The main findings of this study were as follows: 1) in sensitized Brown Norway rat, allergen challenge causes the transient emergence of clustered areas of poor ventilation and significant regional ventilation heterogeneity, as evidenced by high-resolution KES imaging; 2) histological analysis of airway and blood vessel walls, and parenchymal areas showed a higher number of eosinophil and CD68+/H11001 cells, but also higher total cell densities within vs. outside of the VDs; 3) we found a strong correlation between minimum Daw following allergen challenge, a direct in vivo measurement of airway narrowing, and both eosinophil and total cell infiltration within airway walls.

The finding that allergen caused the emergence of clustered defects in ventilation alongside elevations in the overall Newtonian resistance, elastance, tissue damping, and hysteresivity is consistent with our previous findings in OVA-sensitized rabbit (6), and other studies in the literature in both animal models (24) and humans (38). Such defects in regional ventilation can arise from airway constriction, but also luminal obliteration due to mucus secretion and airway wall edema. The fact that the appearance and decay of such VDs was transient in this model suggests that airway constriction was the principal mechanism involved (Fig. 2).

The clustered pattern of the defects suggests that constrictions and closures occurred in small peripheral airways that could not be directly imaged in rat. Theoretical studies using computational modeling have suggested that such patchy VDs can emerge even without heterogeneous airway constriction beyond a critical level of airway smooth muscle activation, due to feedback mechanisms between tidal expansion, resistance, and flow (38–40). However, other local factors may contribute to the heterogeneity of airway narrowing, and both eosinophil and total cell infiltration within airway walls.

Fig. 4. Eosinophil (top), total cell (middle), and CD68+ (bottom) cellular densities in airway wall, vessel wall, and parenchyma within and outside the ventilation defects (VDs). The latter were defined on the basis of the regional concentration of xenon. Data are means ± SE in six sensitized and challenged animals and three controls. *P < 0.05 vs. outside of VDs; #P < 0.05 vs. control.

Fig. 5. Correlation between the minimal cumulated central airway diameter (Daw_{min}) following OVA challenge and mean eosinophil and total cell counts in the airway walls (AW) within the poorly ventilated zones.
in regional ventilation following allergen challenge. We have previously shown that indirect airway smooth muscle stimulation via allergen challenge produced patchy VDs, whereas direct stimulation by methacholine with similar changes in the respiratory mechanical parameters did not (6). Also, uneven deposition of aerosol particles during inhaled challenges can significantly contribute to the patchiness of regional ventilation (6). However, whether the regional distribution of cellular inflammatory effectors can locally affect the probability of VD emergence is not known. Generally, the relation between airway inflammation and bronchial responsiveness in asthma is poorly understood. For example, in clinical studies, blood, sputum, or BAL eosinophilia have shown weak to moderate correlations, at best, with lung function or acute airway responsiveness to allergen (7, 12, 33). Furthermore, trials of monoclonal antibodies blocking interleukin-5 have shown that although blood and sputum eosinophilia were reduced, there were no effects on airway hyperresponsiveness, or early or late response to allergen challenge (7, 12, 33). These findings have questioned the role of eosinophils in airway responsiveness in humans. There is direct evidence in the literature, however, that eosinophils play a prominent role in allergic airway inflammation in asthma (14). Sputum and BAL eosinophilia are hallmark features of atopic asthma, and eosinophils are increased in the airway mucosa of subjects dying of asthma. Moreover, eosinophilic infiltration is present both in central and small peripheral airways of subjects with asthma (26).

Our data suggest that airway inflammation is topographically heterogeneous, and that this phenomenon is associated with the heterogeneity of regional airway constriction and that of regional ventilation in response to allergen. Previous studies in OVA-sensitized mouse using florescence tomography (21) and intravital microscopy (18) have also directly evidenced the spatial heterogeneity of lung inflammation following allergen challenge. We are not aware of any other preclinical studies linking regional inflammation with regional lung function or local airway responsiveness, although positive correlations between overall responsiveness to acetylcholine and both T cell and eosinophilic counts in airway submucosal tissue have previously been demonstrated in OVA-sensitized and exposed Brown Norway rats (13). However, this hypothesis is consistent with recent findings by Harris et al. in subjects with atopic asthma (16). They demonstrated that after segmental allergen challenge, regional ventilation systematically decreased, whereas BAL of the same segment 14 h after challenge showed an inflammatory response and increased eosinophilia, which was not the case in a control lung segment in the same subject. On the other hand, Fain et al. (10) found a significant correlation between the neutrophil content of BAL fluid and the volume of ventilation defects measured using hyperpolarized 3He magnetic resonance imaging in subjects with asthma. These findings highlight the role of local cellular effectors in determining regional airway responsiveness to allergen and its consequences on regional lung function.

In our study, in addition to the local association between cellular infiltration and the emergence of VDs, the strong correlation between eosinophilia in the airway walls within the VDs and central airway narrowing suggests a role for inflammation and eosinophilia in the global airway responsiveness to allergen. The activity exerted by eosinophils is attributed to their production and release of a number of substances such as prestored granular proteins, oxygen radicals, lipid mediators, and numerous cytokines (14). The cytokines produced by these proinflammatory cells can contribute to the enhancement of airway responsiveness through indirect pathways (14). Cationic proteins released from eosinophil granules, such as Major Basic Protein (MBP), have been shown to induce airway hyperresponsiveness following direct tracheal instillation in rat (35) and neutralization of endogenously secreted MBP by a specific antiserum-prevented antigen-induced bronchial hyperresponsiveness (22), which suggests that the activation of eosinophils can locally enhance airway responsiveness. Interestingly, we found a similar relation between proximal airway narrowing and total cell densities. In a previous study in Brown Norway rat, although cyclosporine effectively reduced bronchial eosinophilia, it did not affect airway responsiveness, whereas corticosteroids, which have inhibitory effects on a wider range of inflammatory cells, suppressed both airway hyperresponsiveness and the increase in BAL eosinophils (9). Furthermore, some degree of airway wall thickening due to edema and collagen deposition may have locally contributed to increased airway responsiveness. Together, these data suggest that in addition to the role of eosinophils, other cell types might be involved in determining airway responsiveness in this model of allergic sensitization.

Limitations. There were several methodological limitations in our study. The time resolution of 10 s/image imposed certain limitations to image acquisition. Structural and functional images could be obtained repeatedly in a maximum of only two axial slices. To image the acute response to antigen challenge, which tended to be transitory, we opted to repeatedly image the same axial slice. The stationary, horizontal X-ray beam used for functional imaging in the present study required that the animal be positioned vertically. This posture may have modified the respiratory mechanics parameters. We previously found that, the upright body position in rabbit, is associated with systematic decreases in the respiratory mechanical parameters relative to those obtained with the animals in the supine position (5). However, in the present study, each animal was its own control and changes following allergen challenge were compared with baseline measurements within animals. Furthermore, the presence of ventilation heterogeneities following OVA challenge may bias the assessment of the airway and tissue mechanical changes on the basis of the use of a lumped-parameter model (23). Because this phenomenon is manifested in artifactual increases in G and η, the real tissue effects of OVA on these parameters may have been slightly overestimated.

We counted cellular profiles within histological sections that were selected to match the KES image slice. In this sense, our cellular profile counts do not represent a fair sample of the whole lung. Furthermore, cellular profiles may not reflect accurate absolute counts of cellular elements, because cells with larger cross-sections within the histological preparation have a greater probability of being counted. However, our goal was to compare the cellular profiles within randomly selected regions of interest inside vs. outside of the VDs, assuming a similar counting bias in between the respective regions.

Conclusions. In summary, we have developed an original, high-resolution KES imaging in OVA-sensitized Brown Norway rat to assess the changes in regional lung ventilation
following allergen challenge, and to assess whether local cellular effectors within the airway wall or parenchyma may influence the emergence and spatial distribution of VDs, thereby modulating the dynamic system behavior of the lung during bronchoconstriction. We found that in this model, exposure to inhaled allergen causes the transient emergence of clustered areas of poor ventilation and significant regional ventilation heterogeneity. Histological analysis of airway and blood vessel walls, and parenchymal areas showed higher eosinophil and CD68+ cell counts, but also higher total cell densities within vs. outside of the VDs. We found a strong correlation between minimum Daw following allergen challenge, a direct in vivo measurement of airway narrowing, and both eosinophil and total cell infiltration within airway walls. Our data suggest that airway inflammation is locally heterogeneous, and that this phenomenon is directly involved in determining the heterogeneity of regional airway constriction and that of regional ventilation in response to allergen. This finding brings significant new insight to the current understanding of the emergence of heterogeneous airway constriction, and has important implications for treatment strategies of atopic asthma.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


