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Comparison of early and late responses to antigen of sensitized guinea pig parenchymal lung strips

Tatiana Lanças,¹ David I. Kasahara,² Carla M. Prado,² Iolanda F. L. C. Tibério,² Milton A. Martins,² and Marisa Dolhnikoff¹

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Lanças, Tatiana, David I. Kasahara, Carla M. Prado, Iolanda F. L. C. Tibério, Milton A. Martins, and Marisa Dolhnikoff. Comparison of early and late responses to antigen of sensitized guinea pig parenchymal lung strips. *J Appl Physiol* 100: 1610–1616, 2006. First published January 12, 2006; doi:10.1152/jappphysiol.00828.2005.—The peripheral lung parenchyma has been studied as a component of the asthmatic inflammatory response. During induced constriction, tissue resistance increases in different asthma models. Approximately 60% of the asthmatic patients show early and late responses. The late response is characterized by more severe airway obstruction. In the present study, we evaluated lung parenchymal strips mechanics in ovalbumin-sensitized guinea pigs, trying to reproduce both early and late inflammatory responses. Oscillatory mechanics of lung strips were performed in a control group (C), in an early response group (ER), and in two late response groups: 17 h (L1) and 72 h (L2) after the last ovalbumin challenge. Measurements of resistance and elastance were obtained before and after ovalbumin challenge in C and ER groups and before and after acetylcholine challenge in all groups. Using morphometry, we assessed the density of eosinophils and smooth muscle cells, as well as collagen and elastin content in lung strips. The baseline and postagonist values of resistance and elastance were increased in ER, L1, and L2 groups compared with C ($P \leq 0.001$). The morphometric analysis showed an increase in alveolar eosinophil density in ER and L2 groups compared with C ($P < 0.05$). There was a significant correlation between eosinophil density in parenchymal strips of C, L1, and L2 groups and values of resistance and elastance postacetylcholine ($r = 0.71$, $P = 0.001$ and $r = 0.74$, $P < 0.001$, respectively). The results show that the lung parenchyma is involved in the late response, and the constriction response in this phase is related to the eosinophilic inflammation.

distal lung inflammation; parenchymal oscillatory mechanics; asthma

THE DISTAL LUNG HAS BEEN SHOWN to play an important role in the pathophysiology of asthma. It has been suggested that small airway alterations may significantly contribute to the functional impairment in asthma, especially in the most severely affected patients (4, 9, 18). The peripheral lung parenchyma of asthmatic patients shows eosinophilic inflammation, as well as IL-5 overexpression (1, 11, 19). It has been shown that tissue resistance (R) increases during induced constriction in different animal models of asthma (7, 22, 31, 36). Although the heterogeneity of airway constriction may be responsible for the observed changes in tissue R in response to agonists (17), increases in tissue R may also reflect a direct role of parenchymal tissue in the overall lung response. Lung parenchymal strips submitted to sinusoidal oscillations in vitro can be used to determine the tissue mechanical behavior, as they represent

exclusively the distal units of lung tissue and offer a better assessment of pure tissue properties (6, 13).

Atopic subjects with asthma show bronchoconstriction that usually peaks within 2 h after allergen exposure (early asthmatic response). In ~60% of the subjects, bronchoconstriction also occurs from 3 to 7 h later, characterizing the late asthmatic response, which is associated with airway hyperresponsiveness and eosinophilic airway inflammation (37). This late response is clinically characterized by progressive, persistent, and more severe airway obstruction. In vivo experiments with alveolar capsules in rats have suggested that both airways and peripheral tissue are involved in the two phases of the inflammatory response (21). The involvement of the distal lung in the late asthmatic response could have therapeutic implications, because it has been demonstrated that most of the currently used inhaled steroids are predominantly deposited in the central airways and not in the lung periphery, which may result in the undertreatment of this lung compartment (32).

Hutson et al. (8) described a useful animal model in vivo to study the mechanisms involved in early and late bronchoconstriction responses. They found an early response at 2 h and two late responses at 17 and 72 h after challenge with ovalbumin (OVA) in previously sensitized guinea pigs. Although previous studies had shown that the peripheral lung parenchyma constrict during the early response after in vitro antigen challenge (22), the in vitro mechanical behavior of peripheral lung tissue during the different phases of the inflammatory response in a model of asthma had not been previously reported. In the present study, we evaluated the mechanical properties of lung parenchymal strips in OVA-sensitized guinea pigs, trying to reproduce both early and late inflammatory responses. We also correlated the functional behavior of lung strips with morphometric parameters of eosinophilic inflammation, contractile cell content, and extracellular matrix (ECM) structure.

MATERIAL AND METHODS

This study was approved by the review board for human and animal studies of the School of Medicine of the University of São Paulo. All animals in the study received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication 85-23, revised 1985).

Experimental groups and antigen sensitization. Twenty-four male Hartley guinea pigs weighing 300–400 g were used in the experimental protocol. The animals were divided in four groups: an early response group (ER, $n = 6$); two late response groups (L1, $n = 5$, killed 17 h after the last challenge, and L2, $n = 6$, killed 72 h after the

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last challenge), and a control group (C, $n = 7$). For antigen sensitization, animals were placed in an acrylic box ($30 \times 15 \times 20$ cm) coupled to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil). A solution of OVA (Advanced Nutrition) diluted in 0.9% NaCl (normal saline) was prepared. This solution was continuously aerosolized into the environment until respiratory distress (sneezing, coryza, cough, or retraction of the thoracic wall) occurred, or until 15 min had elapsed. The observer who made the decision to withdraw the guinea pig from the inhalation box was blinded as to the treatment status of the animal. This protocol was repeated twice a week for 4 wk (10, 30) with increasing concentrations of OVA to avoid tolerance. The early response group was exposed to a total of seven inhalations with aerosolized OVA with increasing concentrations from 1 to 5 mg/ml. An eighth challenge with 0.1% OVA was performed directly on lung strips in the organ bath. This optimal dose was determined by preliminary experiments using different OVA doses (0.003–0.3% OVA). The two late response groups (L1 and L2) were exposed to eight inhalations with increasing concentrations from 1 to 10 mg/ml. The control group was submitted to inhalation of aerosolized normal saline.

Oscillatory mechanics evaluation. After sensitization, the guinea pigs were anesthetized with pentobarbital sodium (50 mg/kg ip), tracheostomized, and, after thoracotomy, exsanguinated. The heart and lungs were excised en bloc, and the lungs were infused with Krebs solution (in mM: NaCl, 118; KCl, 4.5; NaHCO_3 , 25.5; CaCl_2 , 2.5; MgSO_4 , 1.2; KH_2PO_4 , 1.2; glucose 10; Sigma Chemical, St. Louis, MO). Sagittal slices of both lungs (here defined as total lung) were fixed in 10% formalin for 48 h, embedded in paraffin, and routinely processed. Subpleural parenchymal strips of the lower lobes ($10 \text{ mm} \times 2 \text{ mm} \times 2 \text{ mm}$) were cut, and the resting length (L_r) and wet weight (W_0) of each strip were measured. Metal clips were glued to either end of the tissue strip with cyanoacrylate. Steel wires (0.5-mm diameter) were attached to the clips; one end was connected to a force transducer (model 404A; Cambridge Technologies) and the other end was connected to a servo-controlled lever arm (model 300B; Cambridge Technologies). The lever arm was capable of peak-to-peak length excursions of 8 mm and length resolutions of $1 \mu\text{m}$ and was in turn connected to a function generator (model 3030; BK Precision, Chicago, IL), which controlled the frequency, amplitude, and waveform of the oscillation. The resting tension (T) was set by movement of a screw thumb wheel system, which effected slow vertical displacements of the force transducer. Length and force signals were converted from analog to digital with an analog-to-digital converter and recorded in a compatible computer. The strips were preconditioned by slowly cycling tension from 0 to 2 g three times and, after the third cycle, they were fixed at 1 g and maintained in the organ bath containing Krebs solution aerated with 95% of O_2 and 5% of CO_2 for 50 min to allow stress relaxation, during which Krebs solution was changed every 15 min. The frequency of oscillation was 1 Hz and the amplitude was 2.5% L_r . After these 50 min, the final resting tension was ~ 0.8 g.

In the control and early groups, the strips were challenged with OVA (0.1%) in the bath to obtain a maximal response (22). After 10 min, Krebs solution was changed before addition of acetylcholine 10^{-3} M (ACh) (Sigma Chemical, St. Louis, MO) to the bath. In the two late response groups, only ACh was added to the bath. Measurements of R and elastance (E) were obtained at baseline for 5 min, after OVA challenge in the ER and C groups, and after addition of ACh to the bath in all groups.

R and E were estimated by the recursive least-squares algorithm to the equation of motion (12):

$$T = E\Delta l + R(\Delta l/\Delta t) + K \quad (1)$$

where T is tension, l is length, $\Delta l/\Delta t$ is the length change per unit time, and K is a constant reflecting resting tension. Results were standardized for strip size. The unstressed cross-sectional area (A_0) of the strip was obtained from the formula:

$$A_0(\text{cm}^2) = W_0/(\rho \times L_r) \quad (2)$$

where ρ is the mass density of the tissue taken as 1.06 g/cm^3 , W_0 is the wet weight in grams, and L_r is the resting length in centimeters. Values of R and E were multiplied by L_r/A_0 . After the mechanical oscillation, the strips were fixed in 10% formalin for 48 h and embedded in paraffin for histological analysis.

Morphometric studies. Five-micrometer-thick slides of both lung strips and total lung were stained with hematoxylin and eosin for routine histological analysis and with Luna staining for eosinophils (15, 34). Lung strips were also submitted to immunohistochemistry with anti-smooth muscle actin and stained with Sirius Red for collagen and Resorcin-Fuchsin staining for elastic fibers (3). For the immunohistochemistry analysis, sections were deparaffinized and a 0.5% peroxidase in methanol solution was applied for 10 min to inhibit endogenous peroxidase activity. Antigen retrieval was performed with citrate solution for 30 min. Sections were incubated with anti-human smooth muscle actin, 1A 4, Dako, Denmark, overnight at 4°C . A LSAB Plus-HRP kit (K-0690 DAKO, Carpinteria, CA) was used as secondary antibody, and 3,3 diaminobenzidine (Sigma Chemical) was used as chromogen. The sections were counterstained with Harris hematoxylin.

By conventional morphometry, we assessed the density of eosinophils in airway walls of total lungs and within alveolar septa of lung strips. Using a 100-point grid with a known area ($62,500 \mu\text{m}^2$ at a $\times 400$ magnification) attached to the microscope ocular, we counted the number of points hitting the outer area of the airway wall (located between the external limit of smooth muscle layer and the adventitia). The airway area in each field was calculated according to the number of points hitting the airway, as a proportion of the total grid area. We then counted the number of eosinophils within that airway wall area. In the lung strips, we counted the number of points hitting alveolar tissue in each field and the number of eosinophils within the alveolar septa. Eosinophil density was determined as the number of eosinophils in each field divided by tissue area. Measurements are expressed as cells per square micrometer. The results were then transformed to cells per square millimeter by adjusting the units (1). Counting was performed in 15 fields of airway walls and 10 fields of lung strip for each animal at a $\times 400$ magnification.

The volume proportion of actin-positive cells in the lung strips was determined by dividing the number of points hitting actin-positive cells by the total number of points hitting the tissue. The volume proportion of collagen and elastic fibers in the alveolar tissue of lung strips was determined by dividing the number of points hitting collagen or elastin by the total number of points hitting alveolar septa. Measurements were performed in 10 fields of lung strip for each animal at a $\times 400$ magnification. Results were expressed as a percentage.

Statistical analysis. Statistical analysis was performed using SPSS 10.0 computer package. Student's t -test was used to compare functional parameters before and after agonist challenge. We used one-way ANOVA and Tukey's post hoc test for comparison of the different parameters among groups. Values are expressed as means $\pm$ SE. Pearson correlation was used for correlations between morphometric and functional parameters. The level of significance was set at $P < 0.05$.

RESULTS

Oscillatory mechanics. Figures 1 and 2 show, respectively, values of R and E (basal and after agonist challenge) in the four groups. We observed an increase in baseline R and E in the three sensitized groups compared with control ($P < 0.001$). After adding agonists to the bath (OVA in the C and ER groups, and ACh in all groups), there was significant increase in R and E values compared with control group ($P \leq 0.001$).

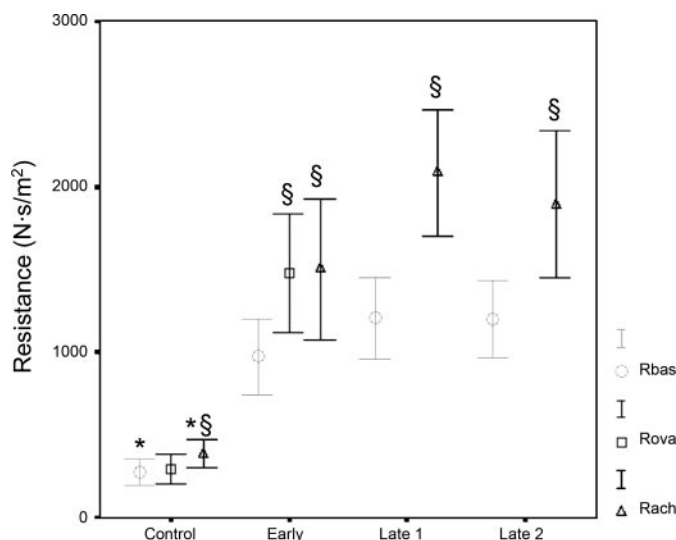


Fig. 1. Means $\pm$ SE of resistance values in parenchymal strips from ovalbumin (OVA)-sensitized and control animals at baseline (Rbas), after OVA challenge (Rova) in control and early response (Early) groups, and after adding ACh 10^{-3} (Rach) in all groups. Late1 and Late2, responses measured 17 and 72 h, respectively, after the last OVA challenge. * $P \leq 0.001$ compared with sensitized groups; § $P < 0.01$ compared with baseline values.

There was also a significant increase in both R and E after agonist challenge when compared with baseline values in all groups ($P < 0.01$). No significant difference in R and E percent increase was observed after agonist challenge among the four groups.

Morphometric analysis. Figures 3 and 4 show, respectively, the eosinophil density in airways and parenchymal strips in the four experimental groups. We observed a significant increase in airway eosinophil density in the three sensitized groups compared with control group ($P < 0.001$). The lung strips showed a significant increase in alveolar eosinophil density in the ER and L2 groups compared with controls ($P < 0.05$).

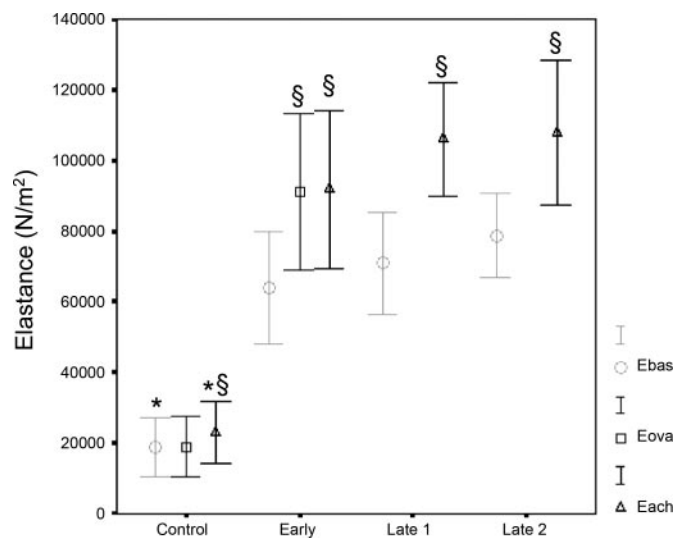


Fig. 2. Means $\pm$ SE of elastance values in parenchymal strips from OVA-sensitized and control animals at baseline (Ebas), after OVA challenge (Eova) in control and early groups, and after adding ACh 10^{-3} (Each) in all groups. * $P < 0.001$ compared with sensitized groups; § $P < 0.01$ compared with baseline values of each group.

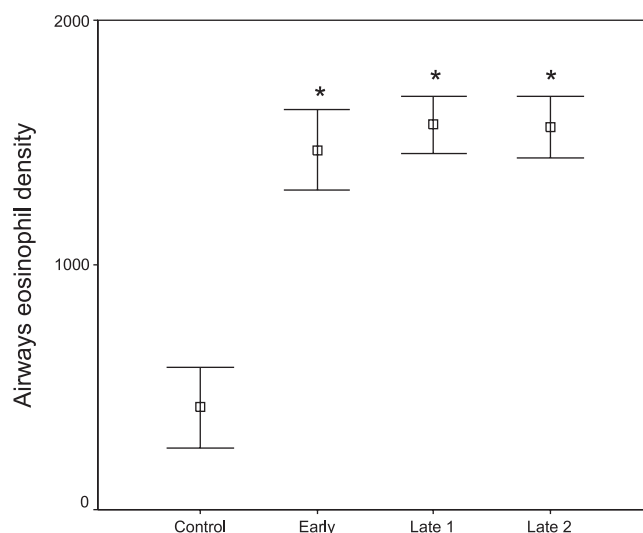


Fig. 3. Means $\pm$ SE of airways eosinophil density in OVA-sensitized and control animals. Results are expressed as cells/mm². * $P < 0.001$ compared with control group.

Figure 5 shows the volume proportion of alveolar collagen in lung strips in control and OVA-sensitized animals. There was a significant increase in collagen content after sensitization ($P = 0.016$). Figure 6 shows representative photomicrographs of alveolar eosinophilic infiltration and collagen density in controls and sensitized animals. There was no significant difference in actin and elastic fiber content in lung strips between control and sensitized animals.

Correlations between morphometric and functional parameters were performed separately for early and late response groups. Table 1 shows the correlation coefficients in ER and C strips, considering the response to OVA and ACh challenge in the bath. We observed significant correlations between the strip actin content and baseline R and E, post-OVA R and E, post-ACh R and E, and post-OVA R percent increase. There was a significant correlation between the percent increase in

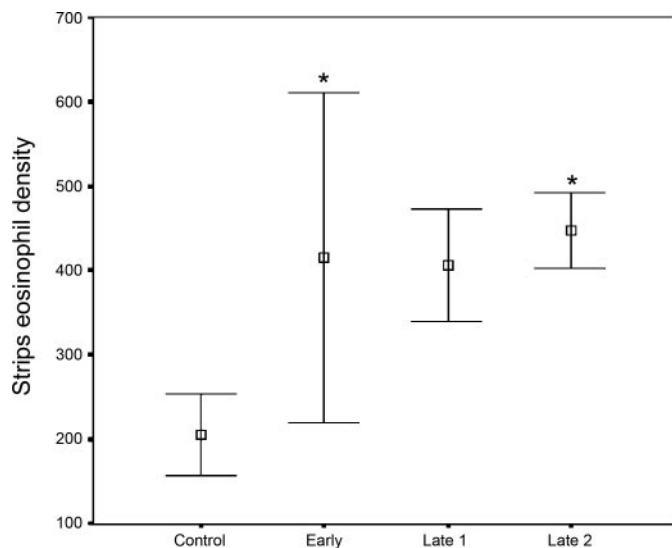


Fig. 4. Means $\pm$ SE of alveolar eosinophil density in parenchymal strips from OVA-sensitized and control animals. Results are expressed as cells/mm². * $P < 0.05$ compared with control group.

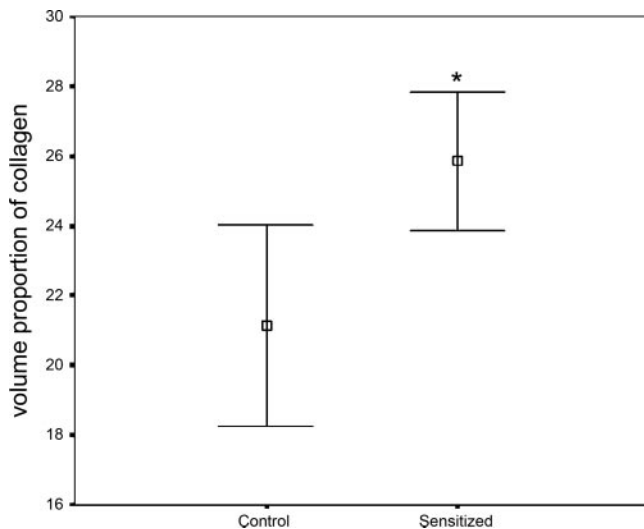


Fig. 5. Means $\pm$ SE of lung parenchymal strips collagen content in OVA-sensitized and control animals. Results are expressed as percentage. * $P = 0.016$ compared with control group.

post-OVA R and the strip eosinophil density. We also observed significant correlations between the strip elastic content and baseline and post-ACh R and baseline, post-OVA, and post-ACh E. There were no significant correlations between collagen content and functional parameters in ER group. Table 2 shows the correlation coefficients in L1, L2, and C strips, considering the response to ACh challenge in the bath. There were significant correlations between eosinophil density and baseline R and E, and post-ACh R and E, indicating that the late response in this asthma model is related to eosinophilic infiltration. We also observed significant correlations between the strip collagen content and baseline R and E and post-ACh

R and E. There were no significant correlations between actin and elastic content and functional parameters in L1 and L2 groups.

DISCUSSION

The present study evaluated the role of peripheral lung parenchyma in the late inflammatory response in an experimental model of asthma. The results show that, in this guinea pig model of chronic allergic inflammation, the lung mechanical and inflammatory responses were present up to 72 h after antigen challenge. The peripheral lung parenchyma was involved not only in the early but also in the late inflammatory response, characterized by the baseline increase of tissue R and E in L1 and L2 groups compared with controls. We also showed that both early and late inflammatory responses presented an eosinophilic infiltration, which was strongly correlated to the late mechanical parenchymal changes. We further demonstrated that this late inflammatory response model is characterized by remodeling of the lung distal units with collagen content increase in sensitized animals.

It has been demonstrated that the airway allergic response can show a late phase that, in most cases, is clinically more severe and more difficult to treat than the early response (23). This late response occurs in ~50% of asthmatic adults (24) and in 70–85% of children with asthma (33).

Although the early response seems to be highly dependent on mediators released by mast cells, mainly represented by leukotrienes and histamine, leading to airway bronchoconstriction and edema, the development of the late phase is associated with the influx and activation of other inflammatory cells in the bronchial mucosa, mainly lymphocytes and eosinophils (25, 26).

The role of peripheral lung tissue in asthma pathophysiology has been intensively studied in the last years. Human studies

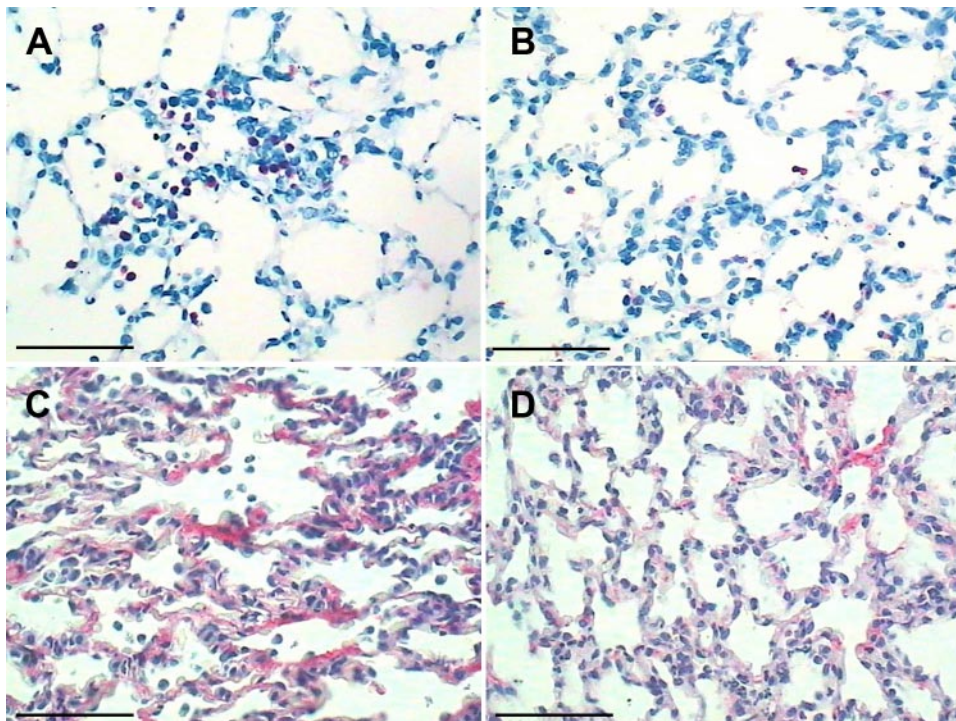


Fig. 6. Photomicrographs of lung parenchymal strip eosinophilic infiltration (A and B) and collagen density (C and D) in controls (right) and sensitized (left) animals. Sensitized animals showed a significant increase in alveolar eosinophilic infiltration and collagen density when compared with controls. Scale bar = 100 μ m.

Table 1. *Correlation matrix between functional and morphometric parameters in parenchymal strips of Control and Early groups (R values)*

MP	Rbas	Rova	%Rova	Rach	%Rach	Ebas	Eova	%Eova	Each	%Each
Eosinophil	0.29 (NS)	0.34 (NS)	0.58 (0.03)	0.24 (NS)	0.10 (NS)	0.30 (NS)	0.32 (NS)	0.47 (NS)	0.30 (NS)	0.11 (NS)
Actin	0.60 (0.02)	0.65 (0.01)	0.61 (0.02)	0.67 (0.01)	0.26 (NS)	0.63 (0.02)	0.66 (0.01)	0.55 (NS)	0.66 (0.01)	0.21 (NS)
Elastin	0.54 (0.05)	0.51 (NS)	0.30 (NS)	0.56 (0.04)	0.20 (NS)	0.58 (0.03)	0.56 (0.04)	0.29 (NS)	0.56 (0.04)	0.02 (NS)

MP, morphometric parameter; Rbas, baseline resistance; Rova, resistance after ovalbumin (OVA) challenge; %Rova, percent increase of resistance after OVA challenge; Rach, resistance after ACh challenge; %Rach, percent increase of resistance after ACh challenge; Ebas, baseline elastance; Eova, elastance after OVA challenge; %Eova, percent increase in elastance after OVA challenge; Each, elastance after ACh challenge; %Each, percent increase of elastance after ACh challenge. *P* values are shown in parentheses; NS, nonsignificant.

show eosinophilic and lymphocytic inflammation and interleukin overexpression in the peripheral lung of asthmatic patients (1, 11, 19). Experimental models and human studies have shown that the distal lung units can respond to agonists with increase in tissue resistance (2, 5). The role of the peripheral parenchyma in the overall lung response to agonists is controversial. It has been demonstrated that changes in tissue resistance after induced constriction are a consequence of the airway inhomogeneities (16, 17). Conversely, other studies show that, even in inhomogeneous lungs, the bronchoconstrictive responses to agonists and inflammation are predominantly located in the lung periphery (31). Nagase et al. (21) evaluated the *in vivo* lung tissue properties in the early and late responses in OVA-sensitized rats with the use of alveolar capsules, suggesting that both airways and peripheral tissue are involved in the two phases of the allergic response. The same authors showed that, after *in vitro* challenge, the peripheral lung parenchyma contracts during the early phase (22). No studies have evaluated the *in vitro* pulmonary tissue behavior at the late asthmatic response. Our results agree with those of Nagase et al. and show that the parenchymal contractile response occurs not only at the early but also at the late phase. Moreover, because there was no difference in the percent increase of R and E after agonist challenge among the groups, we can conclude that the degree of peripheral contractile response is similar in both early and late phases.

In addition to the increase in airway eosinophils in the sensitized groups, we also observed a significant increase in eosinophil density in the peripheral tissue of the ER and L2 groups compared with the C group. This result is similar to human studies that show that lung inflammation in asthma is also present at the alveolar parenchyma level (1, 11, 35). We have recently demonstrated, in humans, that the eosinophilic inflammation is widely distributed within the respiratory tract in fatal asthmatic patients, from the nasal mucosa to the distal lung (1). The observation of peripheral lung inflammation in asthmatic subjects points toward a therapeutic issue, because most of the currently used inhaled steroids are predominantly deposited in the central airways and not in the lung periphery, which may result in undertreatment of this lung compartment

(29). Subjects who develop the late phase of the inflammatory response are usually those who have more severe asthma and therefore require more intensive therapeutic care. In the present study, we demonstrated that there is a contractile response of peripheral lung parenchyma also in this late phase. However, the extent to which our results can be transposed to human asthma is unclear.

In the present study, we observed an eosinophilic infiltration in all sensitized groups (ER, L1, and L2). The positive correlation between R and E values and tissue eosinophil density suggests that the late response and, to a lesser degree, the early response, is at least in part dependent on the eosinophilic inflammation. The mechanisms involved in the tissue behavior in the presence of eosinophilic inflammation have not been fully understood and can involve the response of contractile cells in small airways, alveolar ducts, and alveoli (myofibroblasts) (14). We have previously demonstrated that the peripheral lung tissue response to acetylcholine in humans is not dependent on the presence of airways in the tissue, pointing to a predominant response of alveolar ducts and lung parenchyma (2). Eosinophilic mediators such as LTC₄ (20) and PAF (27) can induce bronchoconstriction. These mediators might also be involved in the alveolar response. It is noteworthy in our study that the tissue response with increase in R and E had a strong correlation with the actin content in the early phase and with the degree of eosinophilic inflammation in the late phase. These results suggest that the early response is more dependent on the presence of contractile cells in the tissue and can be related to acute phase mediators released by other inflammatory cells, such as mast cells, whereas the late phase seems to be dependent on mediators related to eosinophils. The analysis of the inflammatory mediators involved in the two phases of the peripheral parenchyma response to agonists would contribute to a better knowledge of its pathophysiology.

The ECM components determine, to a large extent, the mechanical behavior of lung tissue. Collagen is the most abundant macromolecule of the lung ECM and the most critical for structural integrity. Both collagen and elastin are important in determining lung elastic properties (28). It has been suggested that airway remodeling can be responsible for the

Table 2. *Correlation matrix between functional and morphometric parameters in parenchymal strips of Control, Late 1, and Late 2 groups (R values)*

MP	Rbas	Rach	%Rach	Ebas	Each	%Each
Eosinophils	0.72 (0.001)	0.71 (0.001)	0.30 (NS)	0.75 (<0.001)	0.74 (<0.001)	0.19 (NS)
Collagen	0.82 (<0.001)	0.79 (<0.001)	0.12 (NS)	0.84 (<0.001)	0.83 (<0.001)	0.11 (NS)

P values are shown in parentheses.

persistent obstruction and the pulmonary function decline observed in some asthmatic patients. The small airway remodeling can significantly contribute to the lung function loss in these patients, mainly in those with severe asthma (9, 18). In the present study, we observed peripheral lung tissue remodeling characterized by the significant increase in collagen content in the lung strips of sensitized animals, probably secondary to the chronic inflammation. The collagen content was positively correlated with baseline and post-ACh R and E values in the late-phase groups. Although there was no difference regarding elastic fiber content among the groups, we also observed a correlation between the elastic fiber content and baseline R and baseline and post-ACh R and baseline and post-OVA E values in C and ER groups. Xisto et al. (36) recently demonstrated, in a chronic asthma model in mouse, evidence of lung remodeling not only of the airways but also of the peripheral lung parenchyma, characterized by increase in collagen and actin content in distal lung tissue. We recently described in humans elastic fiber changes in alveolar attachments of distal airways in fatal asthma patients. These peripheral extracellular matrix alterations can be at least in part responsible for the functional alterations observed in these patients, such as loss of deep breath bronchodilator effect in bronchoconstrictive episodes and enhanced airway closure (18). Although the increase in collagen content in the lung periphery has not been described in humans to date, our results reinforce the importance of peripheral lung alterations in asthma, pointing to the remodeling of distal lung units.

In conclusion, we have shown that the peripheral lung parenchyma is involved in the late phase of the inflammatory response in this guinea pig model of chronic allergic inflammation. Lung tissue R and E are increased in the late phase of the response when compared with controls and have similar intensities when compared with the early phase. The degree of antigen-specific response in the early phase is related to the contractile cell content in lung parenchyma. Both early and late inflammatory responses are characterized by eosinophilic inflammation, which seems to be strongly related to the lung tissue response during the late phase. The mechanical behavior of lung tissue in the late phase is also related to the extracellular matrix remodeling, particularly to tissue collagen content.

GRANTS

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