

Oral tolerance attenuates changes in in vitro lung tissue mechanics and extracellular matrix remodeling induced by chronic allergic inflammation in guinea pigs

Adriane S. Nakashima, Carla M. Prado, Tatiana Lanças, Viviane C. Ruiz, David I. Kasahara, Edna A. Leick-Maldonado, Marisa Dolhnikoff, Milton A. Martins and Iolanda F. L. C. Tibério

J Appl Physiol 104:1778-1785, 2008. First published 3 April 2008; doi:10.1152/jappphysiol.00830.2007

You might find this additional info useful...

This article **cites** 46 articles, 22 of which can be accessed free at:

<http://jap.physiology.org/content/104/6/1778.full.html#ref-list-1>

This article **has been cited by** 1 other HighWire hosted articles

Aerobic training reverses airway inflammation and remodelling in an asthma murine model

R. A. Silva, R. P. Vieira, A. C. S. Duarte, F. D. T. Q. S. Lopes, A. Perini, T. Mauad, M. A. Martins and C. R. F. Carvalho

Eur Respir J, May, 2010; 35 (5): 994-1002.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Updated information and services including high resolution figures, can be found at:

<http://jap.physiology.org/content/104/6/1778.full.html>

Additional material and information about *Journal of Applied Physiology* can be found at:

<http://www.the-aps.org/publications/jappl>

This information is current as of December 25, 2011.

Oral tolerance attenuates changes in in vitro lung tissue mechanics and extracellular matrix remodeling induced by chronic allergic inflammation in guinea pigs

Adriane S. Nakashima,¹ Carla M. Prado,¹ Tatiana Lanças,² Viviane C. Ruiz,¹ David I. Kasahara,^{1,3} Edna A. Leick-Maldonado,¹ Marisa Dolhnikoff,² Milton A. Martins,¹ and Iolanda F. L. C. Tibério¹

Departments of ¹Medicine and ²Pathology, School of Medicine, University of Sao Paulo, São Paulo, Brazil; and ³Department of Pathology, The University of Vermont, Burlington, Vermont

Submitted 2 August 2007; accepted in final form 2 April 2008

Nakashima AS, Prado CM, Lanças T, Ruiz VC, Kasahara DI, Leick-Maldonado EA, Dolhnikoff M, Martins MA, Tibério IF. Oral tolerance attenuates changes in in vitro lung tissue mechanics and extracellular matrix remodeling induced by chronic allergic inflammation in guinea pigs. *J Appl Physiol* 104: 1778–1785, 2008. First published April 3, 2008; doi:10.1152/jappphysiol.00830.2007.—Recent studies emphasize the presence of alveolar tissue inflammation in asthma. Immunotherapy has been considered a possible therapeutic strategy for asthma, and its effect on lung tissue had not been previously investigated. Measurements of lung tissue resistance and elastance were obtained before and after both ovalbumin and acetylcholine challenges. Using morphometry, we assessed eosinophil and smooth muscle cell density, as well as collagen and elastic fiber content, in lung tissue from guinea pigs with chronic pulmonary allergic inflammation. Animals received seven inhalations of ovalbumin (1–5 mg/ml; OVA group) or saline (SAL group) during 4 wk. Oral tolerance (OT) was induced by offering ad libitum ovalbumin 2% in sterile drinking water starting with the 1st inhalation (OT1 group) or after the 4th (OT2 group). The ovalbumin-exposed animals presented an increase in baseline and in postchallenge resistance and elastance related to baseline, eosinophil density, and collagen and elastic fiber content in lung tissue compared with controls. Baseline and post-ovalbumin and acetylcholine elastance and resistance, eosinophil density, and collagen and elastic fiber content were attenuated in OT1 and OT2 groups compared with the OVA group. Our results show that inducing oral tolerance attenuates lung tissue mechanics, as well as eosinophilic inflammation and extracellular matrix remodeling induced by chronic inflammation.

experimental model of chronic allergic pulmonary inflammation; eosinophils; collagen and elastic fibers deposition; lung strip mechanics; tolerization

HYPERRESPONSIVENESS, atopy, and chronic eosinophilic and lymphocytic lung inflammation are key features of allergic asthma. Recently, oral tolerance has been recognized as an alternative treatment to autoimmune and allergic diseases (15, 37, 44). In atopic patients, oral, sublingual, or inhaled administration of antigens leads to a reduction in symptoms and decreased local inflammation, as well as a reduction in dyspnea and airway hyperresponsiveness. A recent meta-analysis found that sublingual immunotherapy is beneficial for asthma treatment, albeit the magnitude of the effect is not very large (1, 3, 10, 38, 40).

Previous studies that evaluated animal models of oral tolerance showed an attenuation of airway eosinophilic recruitment, bronchial hyperresponsiveness, and mucous secretion (4, 34, 35). In addition, Russo et al. (33, 34) observed that animals submitted to oral antigen administration protocol presented low levels of T-helper 2 (T_H2) cytokines in bronchoalveolar lavage fluid and a reduction in IgG₁ and IgE ovalbumin-specific antibody production.

The mechanical properties of lung tissue are major determinants of pulmonary physiological function and play an important role in the pathophysiology of asthma, particularly in the most severely affected asthmatic patients (14, 24). Dolhnikoff et al. (7) concluded that human lung tissue strips respond to acetylcholine (ACh) challenge with changes in dynamic mechanical behavior. It has also been shown that tissue resistance increases during induced constriction in different animal models of inflammation (22, 27, 31). In addition, Lanças et al. (17) have recently shown that lung tissue is involved in the late asthmatic response in guinea pigs with chronic allergic lung inflammation, which is correlated to lung tissue eosinophilic recruitment and extracellular matrix (ECM) remodeling.

Inhaled corticosteroids, the gold-standard treatment for asthmatic patients, in its new formulation [hydrofluoroalkane (HFA)] is delivered to distal airways more effectively (68.3%) than chlorofluorocarbon formulations (19.7%) (30). Although eosinophilic infiltration could be adequately controlled in distal airways, it is not clear if both distal lung parenchyma eosinophilic infiltration and ECM remodeling may be sufficiently modulated by this new treatment (2, 25). Considering the increasing evidence supporting the importance of lung peripheral tissue in asthma pathophysiology and the new insights related to oral tolerance as a complementary treatment for this disease, the present study investigates the effects of oral tolerance in distal lung tissue responsiveness, eosinophilic inflammation, and ECM remodeling in animals with chronic allergic pulmonary inflammation.

METHODS

The animals weighed 300–350 g initially and were ~3 wk old. All guinea pigs received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 85-23, Revised 1985), and all experiments de-

Address for reprint requests and other correspondence: I. F. L. C. Tibério, Departamento de Clínica Médica, Faculdade de Medicina da Universidade de São Paulo, Av. Dr. Arnaldo, 455-Sala 1210, 01246-903, São Paulo SP, Brazil (e-mail: iocalvo@uol.com.br or carla@experimental.fm.usp.br).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

scribed in this study were approved by the institutional review board of the University of São Paulo (São Paulo, Brazil).

Experimental model of pulmonary allergic inflammation. Chronic airway inflammation was induced as previously described (29, 41). The guinea pigs were individually placed in a Plexiglas box (30 × 15 × 20 cm) coupled to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil), and an aerosol of ovalbumin solution (grade V, Sigma Chemical, St. Louis, MO) diluted in 0.9% NaCl sterile solution (saline) was generated for 15 min or until respiratory distress occurred. The time that guinea pigs were in contact with aerosol was termed inhalation time. Respiratory distress was defined as the onset of sneezing, coryza, cough, and/or indrawing of the thoracic wall. The observer who made the decision to withdraw the animals from the inhalation box was not aware of the treatment status of the animal. This protocol consists of seven inhalations performed twice a week for 4 wk with increasing concentrations of ovalbumin (1–5 mg/ml) to avoid tolerance (Fig. 1). In inhalations 1–4 (first 2 wk), the dose of ovalbumin used was 1.0 mg/ml. In the 5th and 6th inhalation, animals received a solution with 2.5 mg/ml of ovalbumin, and in the 7th inhalation the dose was increased to 5.0 mg/ml of ovalbumin (20, 28, 29, 41). Control animals received aerosolized saline.

Induction of oral tolerance. Oral tolerance (OT) was induced by offering to the animals 2% of ovalbumin diluted in sterile drinking water (final dilution: 0.02 g/ml) ad libitum for 4 wk beginning concomitantly with the 1st inhalation (OT1 group) or beginning 24 h after the 4th inhalation (OT2 group). Every day we measured the water volume that was drunk by the animals, and the median value was 80 ml of ovalbumin solution during a period of 24 h. The animals of OVA and SAL groups (see *Experimental groups*) consumed the same sterile water volume. The tolerization process was maintained until the end of the experimental protocol (Fig. 1).

Experimental groups. Four groups of guinea pigs were studied: 1) animals that received inhalations with saline and drank sterile water for 4 wk (SAL, $n = 11$); 2) animals that received inhalations with ovalbumin and drank sterile water for 4 wk (OVA, $n = 11$); 3) animals that received inhalations with ovalbumin and drank ovalbumin 2% in sterile water for 4 wk beginning with the 1st inhalation (OT1 group, $n = 11$); and 4) animals that received inhalation with ovalbumin and drank ovalbumin 2% in sterile water for 2 wk beginning after the 4th inhalation (OT2 group, $n = 11$).

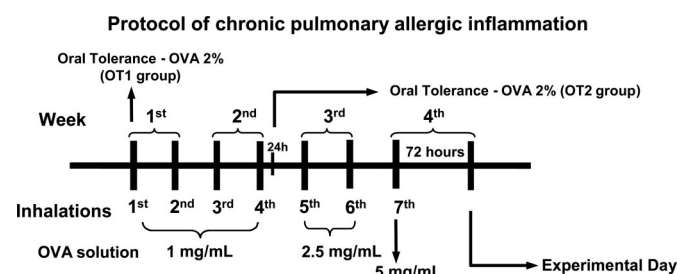


Fig. 1. Timeline of the experimental protocol. Guinea pigs received 7 inhalations (2 per wk during 4 wk) with aerosols of saline (SAL group) or ovalbumin solution (OVA group) with increasing doses of antigen. From the 1st to the 4th inhalations, animals received 1 mg/ml of ovalbumin (2 wk). In the 5th and 6th inhalations (3rd wk), animals inhaled with 2.5 mg/ml of ovalbumin, and in the 7th inhalation (beginning of the 4th wk) the dose of 5 mg/ml of antigen was used. The solution of ovalbumin or saline was continuously aerosolized either for 15 min or until respiratory distress occurred (sneezing, coryza, cough, or retraction of the thoracic wall). Oral tolerance treatment started in ovalbumin-exposed animals together with the 1st inhalation (OT1 group) or 24 h after the 4th inhalation (OT2 group) to avoid an interference with the sensitization process. The oral tolerance was induced by a solution of ovalbumin 2% diluted in drinking water (0.02 g/ml) and offered to guinea pigs ad libitum until the experimental day. Seventy-two hours after the 7th inhalation, all guinea pigs were anesthetized and exsanguinated, and the lungs were removed and submitted to oscillatory mechanics analysis.

Passive cutaneous anaphylaxis. To evaluate if oral-induced tolerance interfered with sensitization to ovalbumin, we measured IgE and IgG₁ antibodies by passive cutaneous anaphylaxis (PCA), as previously described (26, 42). Three guinea pigs from each experimental group were anesthetized with pentobarbital sodium (50 mg/kg ip) on day 28, and 5 ml of blood was obtained by cardiac puncture. Blood was allowed to clot at a temperature of 4°C and centrifuged at 1,000 g for 20 min. For the estimation of anaphylactic IgG₁ antibodies, aliquots of serum were inactivated at 56°C in a water bath for 1 h to neutralize the activity of IgE antibodies. For estimation of IgE antibodies, serum was not heated, and a longer period of sensitization was used. The dorsal region of a normal guinea pig was shaved with an electric hair clipper, care being taken to avoid irritation of the skin. Several subcutaneous injections (100 µl) of serum dilutions (from 1:5 to 1:1,280) were then made on each side of the dorsal skin with a hypodermic needle. After a sensitization period of 24 h for IgG₁ antibodies and 10 days for IgE antibodies, the animals were challenged intravenously with 1 mg of ovalbumin in 0.25% Evans blue solution in saline. Thirty minutes after antigen injection, the animals were euthanized, the skin was inverted, and the lesion diameter was measured on the inner surface of the skin with a transparent ruler. The PCA titer was taken to be the highest dilution that presented a blue spot at least 10 mm in diameter.

Oscillatory mechanics evaluation. Seventy-two hours after the 7th inhalation, eight guinea pigs from each group were anesthetized with pentobarbital sodium (50 mg/kg ip) and tracheostomized, and after thoracotomy they were exsanguinated. The heart and lungs were excised en bloc, and the lungs were intratracheally infused with Krebs solution (in mM: 118 NaCl, 4.5 KCl, 25.5 NaHCO₃, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 10 glucose) (Sigma Chemical). Subpleural tissue strips of the lower lobes (10 mm × 2 mm × 2 mm) were cut. Although guinea pig pleural membrane has contractile cells, it is very difficult to remove it because it is very thin. Thus we decided to maintain the pleura in all animals to avoid a bias in case these membranes were partially removed. The resting length (L_r) and wet weight (W_0) of one strip of each animal were measured. Metal clips were glued to either end of the tissue strip with cyanoacrylate. Steel wires (diameter, 0.5 mm) 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 µm 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 low-pass filtered and converted from analog to digital with an analog-to-digital converter (model SCB-68/16 bits of resolution; National Instruments) and recorded on 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% O₂-5% CO₂ for 50 min to allow stress relaxation. In this period of 50 min called stress relaxation, the Krebs solution was changed every 15 min. The frequency of oscillation and amplitude were adjusted, respectively, to 1 Hz and 2.5% of measured L_r . After these 50 min, the final resting tension was ~0.8 g.

The strips of all animals were challenged with ovalbumin (0.1%) (final dose 0.18 mg/ml) in the bath to obtain a maximal response (17, 27). After 10 min, Krebs solution was changed before adding ACh at 10⁻³ M (final dose: 0.1931 mg/ml) (Sigma Chemical) to the bath. Measurements of tissue resistance and elastance were obtained at baseline for 5 min, and after ovalbumin and ACh challenges in the bath in all experimental groups.

Resistance (R) and elastance (E) were estimated by the recursive least-squares algorithm to the equation of motion (18)

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

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 . Hysteresivity, a dimensionless variable coupling the dissipative and elastic behavior, was calculated by the equation:

$$\eta = (R/E)2\pi f \quad (3)$$

where f is frequency.

Immediately after mechanical oscillation, the strips were fixed in 10% formalin for 48 h, processed, and embedded in paraffin for histological analysis.

Morphometric analysis. A 5- μm -thick slide of lung strips was stained with hematoxylin and eosin for routine histological analysis and with Luna staining for eosinophils (23, 43). Lung tissue 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 (8). For immunohistochemistry, 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) overnight at 4°C . LSAB Plus-HRP kit (K-0690 DAKO, Carpinteria, CA) was used as secondary antibody, and 3,3'-diaminobenzidine (DAB) (Sigma Chemical) was used as chromagen. The sections were counterstained with Harris hematoxylin.

By conventional morphometry, we analyzed the density of eosinophils 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 ocular of the microscope, 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 (17). Counting was performed in 10 fields of lung strip in each animal.

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 in each animal at a magnification of $\times 400$. Results were expressed as a percentage of positive area per total area of tissue.

To ensure the homogeneity of the strips samples used, we quantified the volume proportion of small airways, vessels, and alveolar walls, using the same method of point counting described above, at a magnification of $\times 400$. All morphometric measurements were performed by people blinded to protocol design. The results were expressed as a percentage.

Data analysis. Statistical analysis was performed using SigmaStat software (SPSS, Chicago, IL). The data were examined using ANOVA, the distribution was normal, the variances were homogeneous, and the multiple comparisons were made using Student-Newman-Keuls method (48). Values were expressed as mean and SE and with vertical bar graphics. $P < 0.05$ values were considered significant.

RESULTS

PCA. We observed that sensitized guinea pigs showed an IgG₁ title of 1:1,280. In contrast, sensitized animals submitted to the protocols of tolerization showed a decrease in the IgG₁ titles (OT1, 1:40; OT2, 1:40). In control animals, there was no extravasation of Evans blue dye.

Inhalation time. There was no difference in the inhalation time among the groups studied until the 4th inhalation. In the 5th, 6th, and 7th inhalation, the animals exposed to ovalbumin stayed less time in contact with antigen until respiratory distress occurred, presenting a lower inhalation time during the 7th inhalation (OVA group: $334.63 \pm 26.61 \text{ s}$) compared with saline-exposed animals ($P < 0.05$). Both oral tolerance-treated animals (OT1 and OT2 groups) presented higher inhalation time in the 7th inhalation (OT1 group, $842.25 \pm 41.38 \text{ s}$; OT2 group, $830.0 \pm 61.44 \text{ s}$) compared with ovalbumin-exposed animals that received sterile drinking water ($P < 0.05$ compared with OVA group). There were no significant differences among the oral tolerance groups and saline-exposed animals in all inhalations.

Oscillatory pulmonary mechanics. Figure 2, A and B, respectively, represents the values of resistance and elastance (baseline and after ovalbumin and ACh challenges) in the four

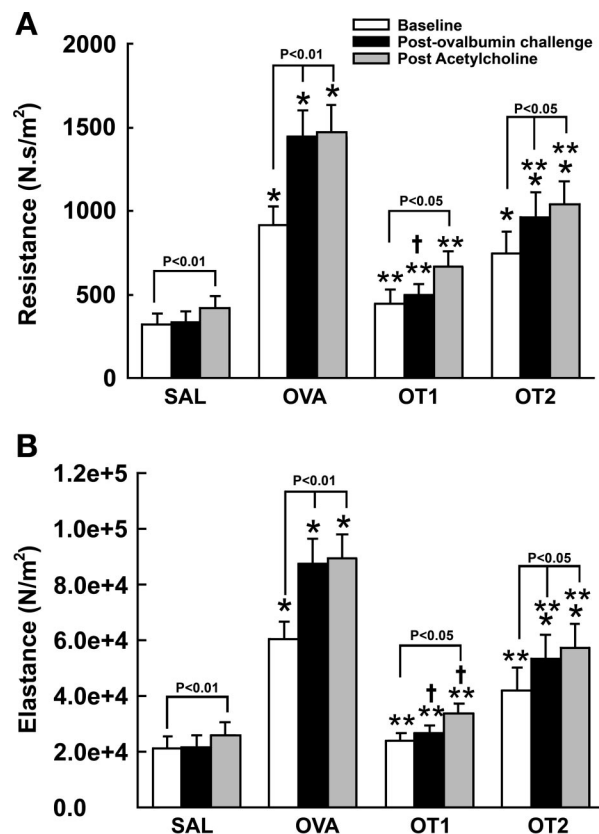


Fig. 2. Mean and SE values of the tissue resistance (A) and elastance (B) obtained at baseline or after challenge with ovalbumin (0.1%, final dose 0.18 mg/ml) and ACh (10^{-3} M , 0.1931 mg/ml diluted in Krebs) in the bath from guinea pigs that previously inhaled saline or ovalbumin and that were treated with oral ovalbumin beginning together with the 1st inhalation or after the 4th. The lines and the respective P values show the comparison between the baseline and the maximal responses in each group. * $P < 0.05$ compared with SAL group, ** $P < 0.05$ compared with OVA group, † $P < 0.05$ compared with OT2 group, respectively.

groups studied. Considering the differences between baseline and maximal responses in each experimental group, we observed in all groups a significant increase in the values of lung tissue resistance and elastance ($P < 0.05$) only after ACh challenge compared with the baseline values. Neither saline group nor OT1 group had a significant increase in lung tissue resistance and elastance after ovalbumin challenge compared with their baseline values.

Comparing the baseline and maximal responses among the experimental groups, we noticed that at baseline and after ovalbumin and ACh challenges the values of tissue resistance and elastance were significantly higher in ovalbumin-exposed animals compared with saline-exposed animals ($P < 0.05$). Both oral-induced tolerance treatments (OT1 and OT2 groups) attenuated the maximal responses of tissue resistance and elastance after ovalbumin and ACh challenges compared with ovalbumin-exposed animals ($P < 0.05$). In addition, after ovalbumin challenge, the OT1 group also presented lower values of tissue resistance and elastance compared with the OT2 group ($P < 0.05$).

Concerning hysteresivity evaluation, there were no differences in either the baseline values or the data obtained after ovalbumin and ACh challenges among the four experimental groups (data not shown).

Morphometric analysis. Ovalbumin-exposed animals had presented significantly increasing eosinophilic inflammation compared with saline-exposed animals ($P < 0.01$). Oral induction of tolerance had decreased recruitment of these inflammatory cells ($P < 0.05$ vs. OVA, Fig. 3), and no difference was observed between OT1 and OT2 groups.

ECM remodeling. Figure 4, A and B, shows, respectively, the volume proportion of alveolar collagen and elastic fiber content in the four groups. We observed that ovalbumin-exposed animals presented an increase in both collagen (Fig. 4A) and elastic fiber content (Fig. 4B) ($P \leq 0.01$) compared with saline-exposed animals. Considering the oral tolerance groups, both treatments (OT1 and OT2 groups) attenuated the deposition of collagen and elastic fibers in alveolar septa ($P < 0.05$) compared with animals exposed to ovalbumin that received sterile drinking water (OVA group).

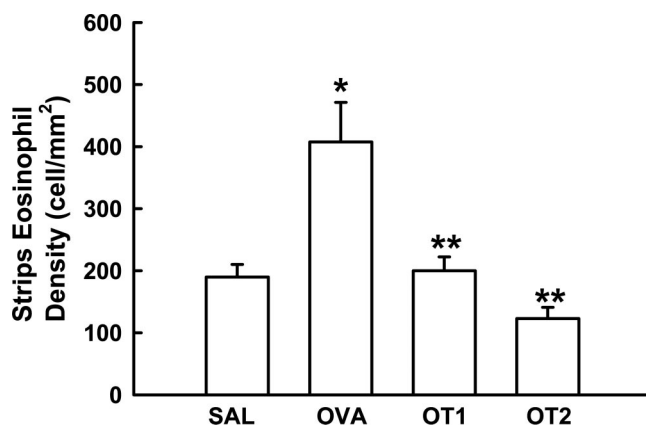


Fig. 3. Mean and SE values of strip eosinophil density from guinea pigs that previously inhaled saline or ovalbumin and that were treated with oral ovalbumin beginning together with the 1st inhalation (OT1 group) or after the 4th (OT2 group). Results are expressed in cells/mm². * $P < 0.01$ compared with SAL group; ** $P < 0.05$ compared with OVA group.

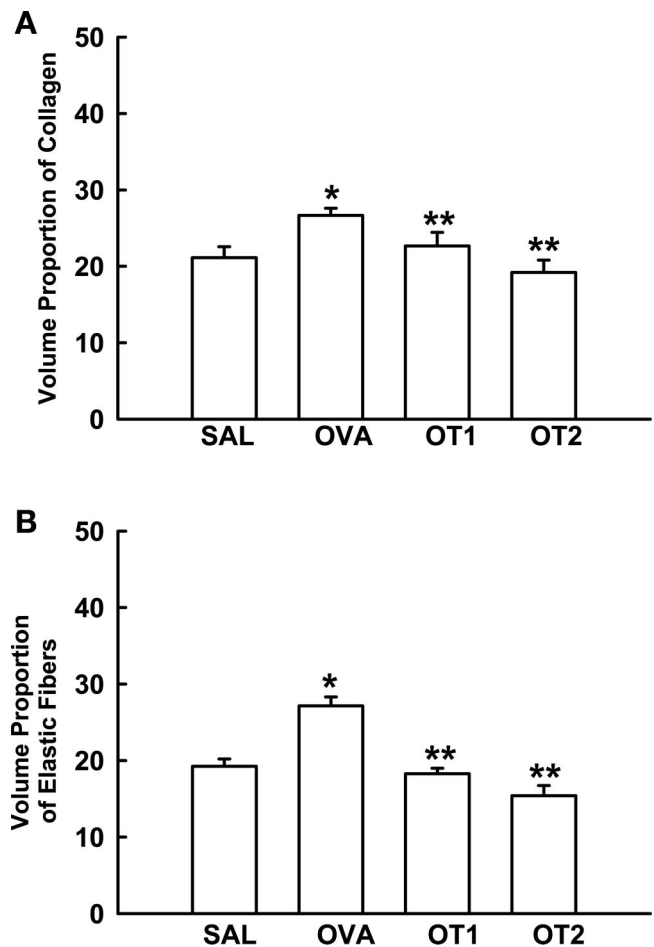
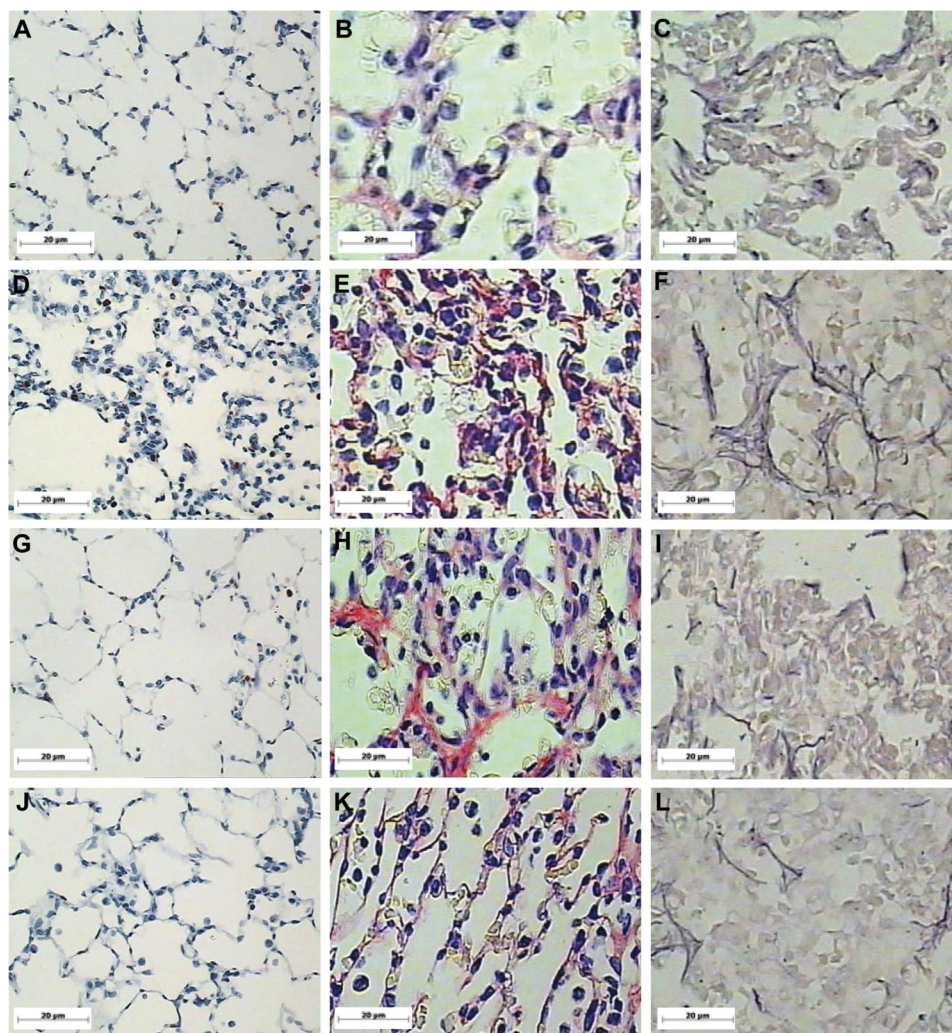


Fig. 4. Mean and SE values of volume proportion of collagen (A) and elastic fibers (B) in strips from guinea pigs that previously inhaled saline or ovalbumin and were treated with oral ovalbumin beginning together with the 1st inhalation (OT1 group) or after the 4th (OT2 group). * $P < 0.001$ compared with SAL group; ** $P < 0.05$ compared with OVA group.

There was no significant difference in the actin content in lung strips among the four experimental groups (SAL group $13.77 \pm 0.69\%$; OVA group $16.06 \pm 1.47\%$; OT1 group $14.78 \pm 1.31\%$; OT2 group $12.81 \pm 1.11\%$). We observed that 60–70% of the fractional area of lung strips was represented by alveolar septa. There were no significant differences in the contents of airways (SAL group $3.97 \pm 0.9\%$, OVA group $2.61 \pm 0.65\%$, OT1 group $1.7 \pm 0.6\%$, OT2 group $1.4 \pm 0.3\%$), vessels (SAL group $1.1 \pm 0.4\%$, OVA group $1.6 \pm 0.4\%$, OT1 group $1.9 \pm 0.0\%$, OT2 group $0.2 \pm 0.1\%$), and alveolar tissue (SAL group $74.4 \pm 2.6\%$, OVA group $52.4 \pm 9.0\%$, OT1 group $68.3 \pm 5.8\%$, OT2 group $74.3 \pm 3.1\%$) among the experimental groups.

Figure 5 shows photomicrographs of lung tissue strips from animals exposed to ovalbumin (OVA group) that show an intense eosinophilic infiltration (Fig. 5D) and increase in collagen density (Fig. 5E) and elastic fiber content (Fig. 5F) compared with saline-exposed animals (SAL group; Fig. 5, A–C). In both groups of induced oral tolerized animals, there was a decrease in the eosinophilic infiltration (OT1, Fig. 5G; OT2, Fig. 5J). Considering the ECM remodeling we noticed that in tolerance animals the collagen (OT1, Fig. 5H; OT2, Fig. 5K) and elastic fiber (OT1, Fig. 5I; OT2, Fig. 5L) contents were

Fig. 5. Photomicrographs of lung parenchymal strips eosinophilic infiltration (A, D, G, J; $\times 400$), collagen density (B, E, H, K; $\times 1,000$), and elastic fibers (C, F, I, L; $\times 1,000$) in saline-exposed (SAL group; A–C), ovalbumin-exposed (OVA group; D–F), ovalbumin-exposed and treated with oral tolerance beginning together with the 1st inhalation (OT1 group; G–I), and ovalbumin-exposed and treated with oral tolerance beginning after the 4th inhalation (OT2 group; J–L). Ovalbumin-exposed animals showed a significant increase in eosinophilic infiltration as well collagen and elastic density compared with saline-exposed animals. Both oral-induced tolerance protocols attenuated all these responses in ovalbumin-exposed animals.



attenuated compared with the ovalbumin-exposed nontolerized guinea pigs.

DISCUSSION

The present study shows that inducing oral tolerance attenuates peripheral lung tissue responsiveness, eosinophilic inflammation, and ECM remodeling in an experimental model of chronic allergic pulmonary inflammation, suggesting that this approach could attenuate or prevent distal lung functional and structural changes induced by chronic allergic inflammation.

Although some studies propose the importance of inducing oral tolerance in the control of asthma pathological alterations and this type of treatment has been used in humans, most of the studies evaluated only the effects on airways. Considering the importance of lung periphery in asthma and the relevant results obtained using immunological tolerance in airways (4, 32, 33, 34, 35), studies that evaluate the efficacy of this treatment in lung parenchyma are relevant. There were no previous studies that evaluated the effects of this strategy in the modulation of lung tissue chronic allergic alterations (4). The sublingual immunotherapy in asthma has been used with beneficial effects, and it is a safe alternative method to the subcutaneous route (3).

Some authors (4, 33, 35) have previously evaluated the effects of oral tolerance in experimental models of airway disease. They observed a reduction in airway inflammation, airway hyperresponsiveness, and mucous secretion induced by chronic inflammation. Although these previous investigations showed an effect of oral tolerance attenuating the airways responses, to our knowledge, the present study has provided the first evidence on the effects of oral tolerance in lung periphery responses in an experimental model of chronic lung inflammation.

We know from previous studies that this experimental model presents some important features of asthma pathophysiology, as intense bronchoconstriction, airways eosinophilic and lymphocytic inflammation, and airway and bronchial vessels remodeling (28, 29, 41). Allergic asthmatics have established antigen-responsive $CD4^+$ T cells in the airways and local lymphoid tissue. Children predisposed to develop asthma often have antibodies to common allergens, indicating that their immune system is already primed to the allergen. Based on animal models of asthma, one might speculate that feeding allergens to allergic asthmatics would increase inflammation, which can be dangerous. Thus animal models might convince us that attempts to stimulate

mucosal tolerance should be used to prevent or treat asthma (5).

The role of lung periphery in asthma pathophysiology has been intensely investigated in the last several years. Several authors have shown that there is an inflammation and remodeling process in lung periphery as well as in central airways (2, 16, 47). The lung periphery strips have been used to study the mechanical and pharmacological properties of the lung periphery (22, 46). We have recently demonstrated that in this experimental model of chronic allergic inflammation, the lung periphery is involved not only in the early but also in the late inflammatory response (17).

The pharmacology industries are pursuing the new technology to develop drugs that also act in lung periphery, and, in this regard, HFA formulation has been shown as a promising medication (25). The effects of HFA have been shown to reduce the peripheral pulmonary inflammation, as previously discussed in a recent study performed by Bergeron et al. (2). They showed that the HFA administration in asthmatic patients did not reduce total collagen deposition and the collagen type III content in peripheral airways. However, the authors did not evaluate the lung parenchyma alterations.

We used two different types of oral tolerance. In the first protocol we began the tolerance induction together with the sensitization protocol. The aim of this protocol is to evaluate a preventive strategy. Our results suggested that this approach is sufficient to avoid the alterations observed in this experimental model. After the 4th inhalation, guinea pigs had already shown pulmonary eosinophilic infiltration, as well as ECM alterations (unpublished results). We had also previously demonstrated (20) that at this moment (after the fourth ovalbumin inhalation) the animals had an increase in the IgG₁ antibody titles (1:320). For this reason, we also evaluated if oral-induced tolerance may be used as a therapeutic approach, reverting the inflammatory and remodeling processes. Then, in the second protocol, the animals received the oral antigen beginning 24 h after the 4th ovalbumin inhalation. Furthermore, we initially evaluated the effects of these protocols of oral-induced tolerance in inflammatory, remodeling, and functional alterations in airways of guinea pigs with chronic allergic inflammation. Ruiz et al. (32) showed that both tolerization protocols attenuated these alterations.

Acute responses to antigen exposure were evaluated by the time that guinea pigs were able to be in contact with the aerosol of ovalbumin (inhalation time). In this context, repeated inhalations of increasing concentrations of ovalbumin progressively reduced these measurements after the 5th inhalation. Both oral-induced tolerance treatments augment the inhalation time in sensitized animals, suggesting that oral tolerance contributes to the control of the acute responses to antigen challenge.

Although *in vivo* apparatus of oscillatory mechanics permits the evaluation of large and small airways, the oscillatory mechanics *in vitro* provides a tool for the specific evaluation of lung periphery with the minimum interference of the compartment represented by the small airways. In addition, we argued that this method *in vitro* permits a specific analysis of the effects of oral tolerance in lung periphery avoiding other compensatory mechanisms that could be activated in *in vivo* studies.

Considering the differences between baseline and maximal responses in each experimental group, we observed in all groups a significant increase in the values of lung tissue mechanics only after ACh challenge compared with the baseline values. It is interesting to notice that neither saline group nor OT1 group had a significant increase in lung tissue mechanics after ovalbumin challenge compared with their baseline values. The comparison among the experimental groups showed that, at baseline and after either ovalbumin or ACh challenges, the values of lung tissue resistance and elastance were attenuated by both oral-induced tolerance treatments (OT1 and OT2 groups) compared with ovalbumin-exposed animals ($P < 0.05$).

These results suggested that oral-induced tolerance treatments were able to attenuate the mechanical changes in lung periphery induced by chronic inflammation, particularly by specific antigen challenge. In these experimental models the tolerization process was antigen specific, and the mechanical responses confirmed this statement.

The animals that received the first protocol presented a lower response after ovalbumin challenge compared with animals that received the second protocol. The differences could be related to the fact that in the first protocol, animals received the oral ovalbumin for 4 wk, although in the second protocol animals received this treatment only for 2 wk. It is interesting to point out that there is no difference between the titles of anaphylactic antibody (IgG₁) between the different protocols used. Both OT1 and OT2 presented the same title of IgG₁ (1:40). Unfortunately, the PCA was a semiquantitative method for antibody title quantification that may not have detected minor differences in antibody titles between tolerized groups. In addition, neither the eosinophilic inflammation nor the collagen and elastin content could explain these differences, since they were similar in both OT1 and OT2 groups.

It is well known that the tolerization process attenuates B-cell responses. Hasegawa et al. (12) demonstrated that B-cells have been implicated in myofibroblast activation mainly by secreting IL-6, IL-9, and fibroblast growth factor. In this regard, it is important to consider that myofibroblasts are one of the contractile elements that modulate lung parenchyma responses (21, 22, 45).

The morphometric analysis of the fractional area of lung constituents within the strips (alveolar wall, blood vessel wall, and bronchial wall) showed that there is no difference in the percentage of each structure in the experimental groups, corroborating the idea that the differences in the contractile responses observed among the experimental groups were not due to a variation in the structural components of the lung studied. Several authors have discussed the importance of these structures in the mechanical behavior of lung tissue, including the consequences of stiffening of the ECM network, as well as of elastin and collagen digestion in these responses (36, 39, 47). We studied strips obtained from subpleural region. In the lung tissue used there was a scanty number of bronchial and blood vessels (<30%). Dolnikoff et al. (7) demonstrated that the human strips responded to contractile stimulation with increases in the dynamic mechanical parameters such as tissue elastance and resistance. These increments were found regardless of whether strips contained or were devoid of small airways, arguing that contractile responses occur at the tissue level.

Recent studies suggest that mechanical properties of lung tissue in baseline conditions may be actually dominated by the connective tissue fiber network, whereas interstitial cells play a less significant role. Romero et al. (31) concluded that pneumoconstriction significantly modifies the intrinsic mechanical properties of the connective matrix by means of a mechanism differing from that of passive stretching. In fact it could be accepted that the contractile cells are able to modulate the mechanical properties of the connective matrix.

The actin content is similar among all experimental groups. This result differs from others that evaluated airway walls and observed an increase in actin content after sensitization (45, 46). Lanças et al. (17), using the same animal model, did not find any differences in the total actin content in saline- and ovalbumin-exposed animals. The authors showed an increase in the lung tissue mechanics after either ovalbumin or ACh challenges. It is important to consider that the actin evaluation was used as a marker of smooth muscle protein, which stained the alveolar duct, airway, and vascular walls, as well as myofibroblasts.

Human studies demonstrated that the eosinophilic infiltration is widely distributed within the respiratory tract, from the nasal mucosa to the distal lung (6, 16). In this experimental model, we previously showed that there is an increase in eosinophils density not only in airways but also in lung tissue (17).

The macromolecules that are most important in determining the mechanical properties of the lung are collagen, elastin, and proteoglycans (39). ECM alterations represent one part of the remodeling process that may have important clinical repercussions on chronic asthmatic responses influencing the decrease in lung function observed in some asthmatic patients (13). However, the intensity of the response of eosinophilic infiltration and ECM remodeling is greater in airway wall than in lung alveolar parenchyma (16, 17, 41). In the present study we observed that animals submitted to chronic inflammatory stimuli have an increase in the lung periphery collagen and elastic fiber content, and the tolerance process attenuated these alterations.

The present data in this article showed that both oral tolerance protocols reduced the eosinophil density as well as the collagen and elastic fiber content in lung parenchyma. Eosinophil-derived cytokines were involved in the modulation of T_H2 responses that trigger macrophage production of transforming growth factor- β_1 , which serves as a stimulus for ECM production (10, 13, 23). Our study corroborates this statement since the reduction in eosinophilic inflammation was associated with a decrease in collagen and elastic fiber content.

In conclusion we observed that oral tolerance attenuates lung tissue hyperresponsiveness, which is associated with attenuation in eosinophil inflammation and ECM collagen and elastic fibers deposition. Although the mechanism involved in these responses need to be further investigated, these findings can open a therapeutic strategy to prevent and/or treat the peripheral lung alterations in asthmatic patients.

ACKNOWLEDGMENTS

We thank Dr. Adenir Perini for assistance in passive cutaneous anaphylaxis technique.

This study was presented in part at the International Meeting of the American Thoracic Society in Orlando, 2004, and at the International Meeting of the European Respiratory Society in Glasgow, 2004.

GRANTS

We are grateful to Brazilian Scientific Agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Programa de Núcleos de Excelência (PRONEX-MCT), and Laboratório de Investigação Médica do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (LIM 20) for financial support.

REFERENCES

1. Abramson MJ, Puy RM, Weiner JM. Allergen immunotherapy for asthma. *Am J Respir Crit Care Med* 160: 1750–1757, 1999.
2. Bergeron C, Hauber HP, Gottfried M, Newman K, Dhanda R, Servi RJ, Ludwig M, Hamid Q. Evidence of remodeling in peripheral airways of patients with mild to moderate asthma: Effect of hydrofluoroalkane-flunisolide. *J Allergy Clin Immunol* 116: 983–989, 2005.
3. Calamita Z, Saconato H, Pela AB, Atallah AN. Efficacy of sublingual immunotherapy in asthma: systematic review of randomized-clinical trials using the Cochrane Collaboration method. *Allergy* 61: 1162–1172, 2006.
4. Chung Y, Cho J, Chang YS, Cho SH, Kang CY. Preventive and therapeutic effects of oral tolerance in a murine model of asthma. *Immunobiology* 206: 408–423, 2002.
5. Cohn L. Food for thought: can immunological tolerance be induced to treat asthma? *Am J Respir Cell Mol Biol* 24: 509–512, 2001.
6. De Magalhães Simões S, dos Santos MA, da Silva Oliveira M, Fontes ES, Fernezián S, Garippo AL, Castro I, Castro FF, Martins MA, Saldiva PH, Mauad T, Dolhnikoff M. Inflammatory cell mapping of the respiratory tract in fatal asthma. *Clin Exp Allergy* 35: 602–611, 2005.
7. Dolhnikoff M, Morin J, Ludwig MS. Human lung tissue responds to contractile stimulation. *Am J Respir Crit Care Med* 158: 1607–1612, 1998.
8. Dolhnikoff M, Mauad T, Ludwig MS. Extracellular matrix and oscillatory mechanics of rat lung parenchyma in bleomycin-induced fibrosis. *Am J Respir Crit Care Med* 160: 1750–1757, 1999.
9. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest* 104: 1001–1006, 1999.
10. Fanta C, Bohle B, Hirt W, Siemann U, Horak F, Kraft D, Ebner H. Systemic immunological changes induced by administration of grass pollen allergens via the oral mucosa during sublingual immunotherapy. *Int Arch Allergy Immunol* 120: 218–224, 1999.
11. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13 α 2 receptor is involved in induction of TGF- β 1 production and fibrosis. *Nat Med* 12: 99–106, 2006.
12. Hasegawa M, Fujimoto M, Takehara K, Sato S. Pathogenesis of systemic sclerosis: altered B cell function is the key linking systemic autoimmunity and tissue fibrosis. *J Dermatol Sci* 39: 1–7, 2005.
13. Holgate S. Mechanisms of allergy and adult asthma. *Curr Opin Allergy Clin Immunol* 1: 47–50, 2001.
14. In't Veen JC, Sterk PJ, Bel EH. Alternative strategies in the treatment of bronchial asthma. *Clin Exp Allergy* 30: 16–33, 2000.
15. Karlsson MR, Kahu H, Hanson LA, Telemo E, Dahlgren UI. Tolerance and bystander suppression, with involvement of CD25-positive cells, is induced in rats receiving serum from ovalbumin-fed donors. *Immunology* 100: 326–333, 2000.
16. Kraft M. Part III: Location of asthma inflammation and the distal airways: clinical implications. *Curr Med Res Opin* 23: S21–S27, 2007.
17. Lanças T, Kasahara DI, Prado CM, Tibério IFLC, Martins MA, Dolhnikoff M. Comparison of early and late responses to antigen of sensitized guinea pig parenchymal lung strips. *J Appl Physiol* 100: 1610–1616, 2006.
18. Lauzon AM, Bates HT. Estimation of time-varying respiratory mechanical parameters by recursive least squares. *J Appl Physiol* 71: 1159–1165, 1991.
19. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Kotliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, Elias JA. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor β 1. *J Exp Med* 194: 809–821, 2001.
20. Leick-Maldonado EA, Kay FU, Leonhardt MC, Kasahara DI, Prado CM, Fernandes FT, Martins MA, Tibério IF. Comparison of glucocorticoid and cysteinyl leukotriene receptor antagonist treatments in an experimental model of chronic airway inflammation in guinea-pigs. *Clin Exp Allergy* 34: 145–152, 2004.
21. Leite-Junior JH, Rocco PRM, Faffe DS, Romero P, Zin WA. On preparation of lung strip for tissue mechanics measurements. *Respir Physiol Neurobiol* 134: 255–262, 2003.

22. Ludwig MS, Dallaire MJ. Structural composition of lung parenchymal strip and mechanical behavior during sinusoidal oscillation. *J Appl Physiol* 77: 2029–2035, 1994.
23. Luna LG. *AFIP Manual of Histologic Staining Methods*. New York: McGraw Hill, 1986.
24. Mauad T, Silva LF, Santos MA, Grinberg L, Bernardi FD, Martins MA, Saldiva PH, Dolnikoff M. Abnormal alveolar attachments with decreased elastic fiber content in distal lung in fatal asthma. *Am J Respir Crit Care Med* 170: 857–862, 2004.
25. Micheletto C, Guerriero M, Tognella S, Dal Negro RW. Effects of HFA- and CFC-beclomethasone dipropionate on the bronchial response to methacholine (Mch) in mild asthma. *Respir Med* 99:850–855, 2005.
26. Perini A, Mota I. Heterogeneity of guinea-pig homocytotropic antibodies. *Immunology* 22: 915–923, 1972.
27. Nagase T, Moretto A, Dallaire MJ, Eidelman DH, Martin JG, Ludwig MS. Airway and tissue responses to antigen challenge in sensitized brown Norway rats. *Am J Respir Crit Care Med* 150: 218–226, 1994.
28. Prado CM, Leick-Maldonado EA, Kasahara DI, Capelozzi VL, Martins MA, Tibério IFLC. Effects of acute and chronic nitric oxide inhibition in an experimental model of chronic pulmonary allergic inflammation in guinea pigs. *Am J Physiol Lung Cell Mol Physiol* 289: L677–L683, 2005.
29. Prado CM, Leick-Maldonado EA, Yano L, Leme AS, Capelozzi VL, Martins MA, Tibério IFLC. Effects of nitric oxide synthases in chronic allergic airway inflammation and remodeling. *Am J Respir Cell Mol Biol* 35: 457–465, 2006.
30. Richards J, Hirst P, Pitcairn G, Mahashabde S, Abramowitz W, Nolting A, Newman SP. Deposition and pharmacokinetics of flunisolide delivered from pressurized inhalers containing non-CFC and CFC propellants. *J Aerosol Med* 14: 197–208, 2001.
31. Romero PV, Zin WA, Lopez-Aguilar J. Frequency characteristics of lung tissue strip during passive stretch and induced pneumoconstriction. *J Appl Physiol* 91: 882–890, 2001.
32. Ruiz VC, Drewiacki T, Leick-Maldonado EA, Prado CM, Nakashima AS, Kasahara DI, Martins MA, Tibério IFLC. Modulation of chronic airway inflammation in guinea pigs by oral tolerance: evaluation of cellular infiltration and peribronchial edema (Abstract). *Eur Respir J* 22: 195S, 2003.
33. Russo M, Jancar S, Siqueira ALP, Mengel J, Gomes E, Ficker SM, Faria AMC. Prevention of lung eosinophilic inflammation by oral tolerance. *Immunol Lett* 61: 15–23, 1998.
34. Russo M, Nahori MA, Lefort J, Gomes E, Keller AC, Rodriguez D, Ribeiro OG, Adriouch S, Gallois V, de Faria AM, Vargaftig BB. Suppression of asthma-like responses in different mouse strains by oral tolerance. *Am J Respir Cell Mol Biol* 24: 518–526, 2001.
35. Sakai K, Yokoyama A, Kohno N, Hamada H, Hiwada K. Prolonged antigen exposure ameliorates airway inflammation but not remodeling in a mouse model of bronchial asthma. *Int Arch Allergy Immunol* 126: 126–134, 2001.
36. Sakai H, Ingenito EP, Mora R, Abbay S, Cavalcante FSA, Lutchen KR, Suki B. Hysteresivity of the lung and tissue strip in the normal rat: effects of heterogeneities. *J Appl Physiol* 91: 737–747, 2001.
37. Smith KM, Eaton AD, Finlayson LM, Garside P. Oral tolerance. *Am J Respir Crit Care Med* 162: 175–178, 2000.
38. Sopo SM, Macchiaiolo M, Zorzi G, Tripodi S. Sublingual immunotherapy in asthma and rhinoconjunctivitis; systematic review of paediatric literature. *Arch Dis Child* 89: 620–624, 2004.
39. Suki B, Ito S, Stamenovic D, Lutchen KR, Ingenito EP. Biomechanics of the lung parenchyma: critical roles of collagen and mechanical forces. *J Appl Physiol* 98: 1892–1899, 2005.
40. Tari MG, Mancino M, Monti G. Efficacy of sublingual immunotherapy in patients with rhinitis and asthma due to house dust mite. A double-blind study. *Allergol Immunopathol* 18: 277–284, 1990.
41. Tibério IFLC, Turco GMG, Leick-Maldonado EA, Sakae RS, Paiva PSO, Warth MPTN, Lapa e Silva JR, Saldiva PHN, Martins MA. Effects of neurokinin depletion on airway inflammation induced by chronic antigen exposure. *Am J Respir Crit Care Med* 155: 1739–1747, 1997.
42. Warth MPTN, Maldonado EA, Fernezlian SM, Leme AS, Perini A, Saldiva PH, Martins MA. Neurokinin depletion attenuates pulmonary changes induced by antigen challenge in sensitized guinea pigs. *Am J Physiol Lung Cell Mol Physiol* 268: L781–L788, 1995.
43. Watanabe T, Okano M, Hattori H, Yoshino T, Ohno N, Ohta Sugata N, Takai T, Nishizaki K. Roles of FcγRIIB in nasal eosinophilia and IgE production in murine allergic rhinitis. *Am J Respir Crit Care Med* 169: 105–112, 2004.
44. Weiner HL. Oral tolerance: immune mechanisms and treatment of auto-immune diseases. *Immunol Today* 18: 335–343, 1997.
45. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 214: 199–210, 2008.
46. Xisto DG, Farias LL, Ferreira HC, Pincanço MR, Amitrano D, Lapa e Silva JR, Negri EM, Carnielli D, F Silva, LF, Capelozzi VL, Faffe DS, Zin WA, Rocco PM. Lung parenchyma remodeling in a murine model of chronic allergic inflammation. *Am J Respir Crit Care Med* 171: 829–837, 2005.
47. Yuan H, Kononov S, Cavalcante FSA, Lutchen KR, Ingenito EP, Suki B. Effects of collagenase and elastase on the mechanical properties of lung tissue strips. *J Appl Physiol* 89: 3–14, 2000.
48. Zar JH. *Biostatistical Analysis*. Englewood Cliffs, NJ: Prentice-Hall, 1984, p. 206–235.