

**INVESTIGATION OF AIRWAY INFLAMMATION AND  
ASTHMA BY REPEATED BRONCHOALVEOLAR LAVAGE  
COMBINED WITH MEASUREMENTS OF AIRWAY  
AND LUNG TISSUE MECHANICS IN INDIVIDUAL RATS**

Summary of Ph.D. Thesis

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## SUMMARY

Acute and chronic airway inflammations are the main pathogenetic features of numerous pulmonary diseases. There are several methods studying the pathomechanisms of inflammatory respiratory diseases. To assess the severity of lung diseases, the bronchoalveolar lavage (BAL) and mechanical lung function tests are the most current diagnostic methods in the experimental and human pulmonology. However, repetition of BAL procedures and assessments of respiratory mechanic parameters in small rodents (mice and rats) mostly are not allowed, animals are regularly sacrificed at the end of the experiments. For that reason there is no possibility for serial assessments, longitudinal follow-up of pathological changes and comparison of results within the same animals.

In the present study there is exhibited an individual animal model that provides follow-up of bronchoalveolar lavage fluid (BALF) with analysis of cellular profile, as well as measurements of respiratory mechanics. BALF collections were achieved by methods of repeated partial BAL. Separately measured airways and lung tissue mechanics were assessed with low frequency forced oscillation technique (FOT) in individual rats. Longitudinal changes are detected within the same animals in BALF cellular profile and lung tissue mechanics by induction of an acute lung injury (ALI) with an intraperitoneal injection of *E coli* lipopolysaccharide (LPS). Bronchial hyperreactivity (BHR) to exogenous constrictor stimuli (metacholin) is assessed and the influx of cells into the lungs repeatedly in rats exposed to different modes of administration of the allergen, ovalbumin (OVA). Furthermore, histopathological consequences of lung tissue followed by LPS and OVA expositions are identified.

The applied method allows longitudinal follow-up of the BALF cellular profile and airway and lung tissue mechanics in rats. Subsequent systemic administration of LPS, makes the early detection of ALI possible in the BALF and respiratory mechanics. Following single systemic administration combined with chronic inhalation of OVA, the self-controlled study design provides experimental evidence of the strong association between the BHR and the number of eosinophils in the BALF. On the basis of histopathological results, the LPS induced rat model is not only suitable for the investigation of ALI/ARDS, but also allows an assessment of a chronic inflammatory process leading to bronchus associated lung tissue (BALT) hyperplasia and emphysema. Furthermore, following the OVA sensitisation a chronic inflammation with allergic characterisation is revealed in the rat lung tissue.

In conclusion, these animal models may be feasible to research of experimental ALI/ARDS, BALT, emphysema and asthma bronchiale.

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8. Bánfi A., Peták F., Székely E., Kósa L., Bari F., Tóth-Szúki V., Novák Z., Kísérletes asztma modell túlélő patkányokon (oral presentation in Hungarian). Annual Meeting of Association of Paediatric Pulmonology, Győr, Hungary, November 8-10, 2007

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I. Andrea Bánfi, László Tiszlavicz, Edgár Székely, Ferenc Peták, Valéria Tóth-Szüki Levente Baráti, Ferenc Bari, Zoltán Novák, Development of Bronchus-Associated Lymphoid Tissue Hyperplasia Following Lipopolysaccharide-Induced Lung Inflammation in Rats *Experimental Lung Research*, 35:186-197, 2009, IF: 1.618 (2008)

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#### PRESENTATIONS RELATED TO THE TOPIC OF THE PH.D. THESIS

1. Bánfi A., Peták F., Székely E., Kósa L., Bari F., Tóth-Szüki V., Novák Z., Ismételt broncholaveolaris lavage (BAL) és légzésmechanikai vizsgálatok (FOT) túlélő patkányon (oral presentation in Hungarian). Annual Meeting of Hungarian Association of Bronchology, Lillafüred, October 8-9, 2004

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## ABBREVIATIONS

ALI, acute lung injury  
ANOVA, analysis of variances  
ARDS, acute respiratory distress syndrome  
BAL, bronchoalveolar lavage  
BALF, bronchoalveolar lavage fluid  
BALT, bronchus associated lymphoid tissue  
BHR, bronchial hyperreactivity  
CTRL., control  
DAD, diffuse alveolar damage  
E. coli, Escherichia coli  
ET, endotracheal  
FOT, forced oscillation technique  
G, tissue damping  
H, elastance  
H&E, haematoxylin and eosin  
I, inertance  
Iaw, inertance of the airways  
ID, internal diameter  
ip, intraperitoneal  
iv, intravenous  
KCl, potassium-chloride  
LM, light microscopy  
LPS, lipopolysaccharide  
MCh, metacholin  
OD, outer diameter  
OVA, ovalbumin  
PAS, periodic acid-Schiff  
P1, lateral pressures at the loudspeaker end  
P2, lateral pressures at the tracheal end  
PE, polyethylen  
R, resistance  
Raw, resistance of the airways  
SE, standard error  
TC, total cell count  
Zrs, respiratory system impedance  
 $\eta$ , respiratory tissue hysteresivity

## 1. INTRODUCTION

Inflammatory diseases of the lung concern a substantial proportion of the population of the world. Production of cellular and non-cellular elements of the airway inflammation against to different agents - pathogens, allergens and other irritants - play an important role in the modulation of acute and/or chronic airway inflammation. While acute inflammation is an immediate defensive reaction followed by repair processes that restore the lung tissue back to normal, chronic inflammation persists for a long time. Chronic inflammation represents the pathological basis of several pulmonary diseases, like asthma, chronic obstructive pulmonary disease, mucoviscidosis bronchiectasis etc. Pathogenesis of chronic airway inflammation is not clear, however, there are evidences suggesting the involvement of many exogenous and endogenous factors. Development of chronic airway inflammation is not fully characterized, but there is evidence that it may begin in infancy or childhood. Inflammatory processes of the respiratory tract occur in the mucous membrane and in the bronchoalveolar surface. Investigation of pathomechanisms of respiratory diseases as well as cellular and/or soluble elements is possible by bronchoalveolar lavage (BAL) procedure in pulmonology and also in experimental animals. Clinical longitudinal studies to explore the long-term development of a pulmonary disease process or the effects of different drugs or toxic substances are frequently performed in order to follow the changes in the mechanical properties of the respiratory system.

### *1.1. Repeated bronchoalveolar lavage combined with lung mechanics measurements in individual rats*

Measurements of airway responsiveness and BAL for the assessment of cellular and chemical profiles are commonly combined in human subjects and animal models. However, the size of the lungs in humans and in larger animals allows repetition of the BAL procedure, small rodents (mice and rats) are regularly sacrificed at the end of the experiments. Accordingly, there is no possibility for repeated interventions; independent groups are used for these experiments and the changes in the lungs are detected from unpaired comparisons. Nevertheless, because of the baseline variability and/or the apparent scatter in the lung responsiveness, large numbers of animals are required in the independent groups. The ability to successfully intubate the trachea of small rodents and control their ventilation is therefore important for longitudinal studies, in which recovery from anaesthesia and repeated measurements of airway and parenchymal mechanics and BAL are required. There has been a number of reports in the literature regarding repeated intubation, lung mechanical measurements and BAL in mice and rats. Reports about methods, which allow the follow-up of the BAL fluid profile and the airway and tissue mechanics in individual rodents are relatively few and generally the methods are not focused for recovery of animals after investigating procedures.

### *1.2. Acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS)*

ALI and/or ARDS are caused by a variety of unrelated insults, including infection with Gram-negative bacteria. They are characterised by an acute inflammatory process in the air spaces and lung parenchyma. The loss of barrier function of the alveolar epithelial and pulmonary capillary endothelial cells results in respiratory failure in critically ill patients. A huge amount of pro- and anti-inflammatory cytokines and chemokines play role in mediating, amplifying, and perpetuating inflammatory-induced acute lung injury, which manifest clinically in pneumonia, sepsis, and shock syndrome. The term of ALI should

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the pulmonary consequences of a chronic inflammatory process leading to BALT hyperplasia and emphysema. The OVA treatment resulted in general chronic inflammation with allergic characterisation in rat lung. This result corresponded with earlier experimental and human histological investigations.

In this way, our individual rat model could be expendable to further follow-up of chronic respiratory diseases, including asthma bronchiale.

apply to a broad range of pathological abnormalities in the lung, and the term of ARDS should be reserved for the most severe cases of acute lung injury.

Endotoxin-induced lung inflammation can be performed especially well by the systemic or intratracheal administration of the bacterial endotoxin, mainly *Escherichia coli* (*E.coli*) LPS in animal models. There have been several literature reports on the pathological histology following the LPS-induced inflammatory response in the lung tissue. Some of these studies revealed the participation of various cells (neutrophils, macrophages, and lymphocytes) and signs of diffuse alveolar damage (oedema, alveolar hyperaemia, haemorrhage, type II pneumocyte alterations, alveolar macrophage accumulation, and the development of hyaline membrane). Furthermore, other authors have reported that LPS may induce additional pathological alterations in the lungs, with hyperplasia of bronchus associated lymphoid tissue (BALT) in small rodents and a single episode of *E. coli* endotoxaemia causes a multiphase alveolar inflammatory response (neutrophil influx and recovery). In addition, emphysema and bronchial mucous cell hyperplasia is induced by tracheal or intraperitoneal LPS administration to rodents. The wide variety of histopathological changes observed in the lungs following LPS administration indicates that the morphological changes in the lung parenchyma have not been characterised completely.

### ***1.3. Asthma bronchiale***

On the basis of actual conceptions, asthma bronchiale is a chronic inflammatory lung disease, which characterised by infiltration of inflammatory cells (mostly eosinophils) into the respiratory mucosa leading to enhanced reactivity of the airways to various constrictor stimuli. There is a correlation between eosinophil counts in BAL fluid and the severity of bronchial hyperreactivity (BHR).

Although the underlying pathophysiological mechanisms responsible for BHR have not been fully elucidated, animal models have contributed substantially to the understanding of this lung disease by demonstrating the key role of airway inflammation, following exposure to various allergens. Exposure to materials such as ozone, ragweed, ascaris suum extract or most commonly ovalbumin (OVA) leads to chronic airway inflammation in animal models. Since such exposure causes an influx of various inflammatory cells into the mucosa and lamina, the inflammatory process in the airways has been assessed by analysing the cellular content of the bronchoalveolar lavage fluid (BALF). BHR has been characterised via the changes in lung responsiveness to non-specific constrictor stimuli, such as histamine, exercise, adenosine and methacholine (MCh). Since the immune responses of animals exhibit considerable inter-individual variability, the performance of longitudinal studies with repeated measurements of the airway and tissue mechanics and the profiling of BALF are particularly important following allergic sensitisation. Various sensitisation protocols have been applied in different animal models, with variable results, and the differences between these methods have not been fully characterised.

## 2. AIMS

The specific objectives of the present study were:

2.1. To develop an individual animal model that provides follow-up of BAL fluid cellular profile, as well as the airway and tissue mechanics by methods of reproducible, partial BALF collections and separately measured airways and lung tissue mechanics with low frequency forced oscillation technique (FOT) in individual rats.

2.2. To detect the longitudinal changes within the same rat in BALF cellular profile and lung tissue mechanics:

- by induction of a mild ALI with an intraperitoneal injection of *E coli* lipopolysaccharide
- to assess the bronchial hyperreactivity and the influx of cells into the lungs repeatedly in rats exposed to different modes of administration of the allergen, OVA.

2.3. To identify the histopathological consequences of lung tissue followed by LPS and OVA expositions in rats.

## 3. MATERIALS AND METHODS

### 3.1. Animal preparations

During experiments male Wistar rats (weight range: 350-500 g) were studied. Anaesthesia was induced with an intraperitoneal (ip) injection of 5% chloral hydrate (400 mg/kg). This dose can keep rats fully anaesthetised for 50–60 min. For the intubation the rat was suspended at an angle of 45° by its two front upper teeth, by a rubber band attached to a Plexiglas support. A 150-W halogen light source (Nicon Volpi Cold Light Illuminator) with two flexible fibre-optic arms allowed transillumination of the trachea just below the vocal cords. During this direct visualization, a 7.0-cm-long (ID 1.5 mm, OD 2.0 mm) polyethylene (PE) catheter was inserted with the help of a Draeger baby laryngoscope into the trachea via the oral cavity. The rat was then removed from the Plexiglas support, placed in a supine position on a special holder, attached to a small animal ventilator (Model 683, Harvard Apparatus, South Natick, MA, USA), and mechanically ventilated with room air (70 breaths/min, 7 ml/kg tidal volume). The tail vein was cannulated with a 24-gauge cannula (Vygonüle V 24G) and muscle relaxation was achieved by administering pancuronium bromide (0.2 mg/kg) intravenously (iv).

### 3.2. Measurement of respiratory mechanics

The input impedance of the respiratory system ( $Z_{rs}$ ) was next measured during short end-expiratory pauses interposed in the mechanical ventilation. This measurement set-up was used to collect  $Z_{rs}$  data. Briefly, a three-way tap was used to switch the ET tube from the ventilator to a loudspeaker-in-box system at end-expiration. The loudspeaker delivered a computer-generated small-amplitude (<1 cmH<sub>2</sub>O) pseudorandom signal (23 non-integer multiples between 0.5 and 21 Hz) through a 100-cm-long, 2-mm-ID polyethylene tube. Two identical pressure transducers (model 33NA002D, IC Sensors, Malpitas, CA, USA) were used to measure the lateral pressures at the loudspeaker end ( $P_1$ ) and at the tracheal

insults depend on several factors (species, dose, mode of delivery, timing, type of toxin, etc.). The current findings have a potential for developing an approach that allows fully automatic measurement of BALT and inflammatory changes by combining the present technique with recent quantitative methods. The present rat model is not only suitable for the investigation of acute alterations, such as ALI/ARDS, but also allows an assessment of the pulmonary consequences of a chronic inflammatory process leading to BALT hyperplasia and emphysema. OVA challenge caused chronic bronchitis with allergic manifestations by histopathology. After OVA treatments, all of the inflammatory cells were increased in the lung tissue not only peribronchial, but perivascular localisation. These results parallel with literature data, in which similar inflammatory cell invasions were reported in OVA sensitised rodents (mice and rats). However, in our study the number of the eosinophil cells was not significantly more, compared with other cell types including lymphocytes and plasma cells. This result is acceptable, because continuous human and experimental histopathologic investigations evidenced that the presence of cellular populations in the asthmatic airways is a result of a general inflammatory response caused by various cell types. Renzi and co-workers examined the inflammatory cell populations in the airways and parenchyma after antigen challenge in the rat. They have found that the repeated antigen challenge significantly increased the cellular yield (mostly neutrophils) from the large and small airways and lung parenchyma.

Particularly notable are the data of Çokugras and co-workers who examined biopsy specimens from children. They registered eosinophilia in lung tissue in one case of ten. In the other nine cases, the submucosa was infiltrated with lymphocytes.

As many opened question are to relate in nature procession of asthma, these individual asthmatic rat model may serve the research of acute — as well as late phase of asthma (remodelling, change of smooth muscle alterations, etc), not only in point of BAL fluid cellular profile and lung tissue mechanics, but also in histopathology.

## 6. CONCLUSIONS

In conclusion, a method has been developed that allows follow-up of the BAL fluid cell count and airway and tissue mechanics in rats without euthanising the animals. There was no long-term effect of the repeated measurement techniques on the cell number, on the cellular profile or in the respiratory mechanics.

By application of the prepared method, the systemic administration of LPS allows the early detection of ALI in the BAL cellular profile and respiratory mechanics, even in a relatively mild form. Moreover, the combination of BAL and noninvasive assessment of airway and tissue mechanics permits the detection of emphysematous parenchymal damage without the need for a terminal histological investigation.

The follow-up of the BALF cell counts and airway and tissue mechanics in rats provides the effects of different OVA treatment protocols on the lung responsiveness and on the inflammatory markers within the same rat. Repeated ip injections of OVA alone or its combination with a single inhalation of the allergen do not alter the lung reactivity to MCh and have no effect on the BALF cellular profile. However, following chronic inhalation of OVA, the self-controlled study design provided experimental evidence of the strong association between the airway hyperreactivity to exogenous constrictor stimuli and the number of eosinophils in the BALF.

On the basis of histopathological results, the LPS induced rat model is not only suitable for the investigation of acute alterations, such as ALI/ARDS, but also allows an assessment of

do not play a role in the effectiveness of OVA sensitisation, i.e. BHR or a change in the BALF cellular profile was not detectable 14 or 20 days after the first OVA injection. The wide variety in the experimental models used in these previous studies may explain this controversy. It is possible that ragweed and grass pollen extracts induce stronger stimuli to the immune system than does OVA. Furthermore, lung inflammation and BHR have been provoked by a single OVA aerosol in highly inbred Brown-Norway rats. Since genetic differences influence the expression of a complex immune disease, it seems plausible that the Wistar rats used in the present study do not develop a detectable BHR or airway inflammation after a single OVA inhalation in contrast with a more susceptible immune system in Brown-Norway rats.

Previous results obtained following multiple inhalational challenges with an allergen are not much more uniform either. Lack of effects, massive BHR and/or airway inflammation, or the absence of BHR despite a massive inflammatory cell influx have all been reported following chronic inhalation of the allergen in various mammals. It is noteworthy, however, that while the chronic OVA inhalation in Group 4 affected the BALF parameters markedly, its effects on the MCh-induced airway responses could be statistically detected only by applying a statistical test utilising a repeated experimental design. This finding demonstrates the need for the repeated study design to clarify the consequences of allergen exposure that obviously generates highly variable changes in the immune system of the individual animals. It has been well established that antigen stimuli recruit and activate eosinophils. These cells play a key role in the pathogenesis of BHR by producing bronchoactive mediators, which participate in the development of airway inflammation. Repeated measurements within the same rat revealed that the decreases in PC<sub>100</sub> and the increase in the number of eosinophils were always associated in the animals in Group 4 where BHR developed. This finding confirms that in the current model of BHR, repeated antigen inhalation acts as a chronic stimulus for inflammatory cell infiltration into the airways. This phenomenon was manifested in marked eosinophilia, similar to results observed in human subjects with asthma, which was clearly detectable in the repeated partial BALF. Repeated ip injection of the allergen alone or its combination with a single inhalation challenge with OVA was not sufficient to trigger such a mechanism in the present animal model. Although this observation is in contrast with previous results demonstrating eosinophil influx and BHR even after a single aerosol OVA exposure, the differences in species and in rat strains may readily explain this apparent controversy, as described above.

- Our morphological results demonstrated that the BALT hyperplasia was the most characteristic histopathological finding beside the signs of different forms of DAD (the obvious manifestations of ALI/ARDS) and emphysema after systemic LPS administration.
- Combination of an ip OVA injection and repeated OVA inhalations caused chronic inflammation by histopathology not only around peribronchial but perivascular localisations.

Examinations of lung tissue specimens from intraperitoneally administered LPS rats showed two characteristic histological alterations. Predominantly BALT hyperplasia was detected, together with the less characteristic DAD. Additionally, we observed emphysema in almost all animals. These two different histopathological entities (DAD and BALT) indicate that a double reaction may develop in response to one stimulus. We anticipate that the responses to endotoxin antigen

end (P<sub>2</sub>) of the wave-tube. The signals P<sub>1</sub> and P<sub>2</sub> were low-pass filtered (5th-order Butterworth, 25-Hz corner frequency), and sampled with an analogue-digital board at a rate of 256 Hz. Fast Fourier transformation with 4-s time windows and 95% overlapping was used to calculate the pressure transfer functions (P<sub>1</sub>/P<sub>2</sub>) from the 6-s recordings collected during apnea. Z<sub>rs</sub> was calculated as the load impedance of the wave-tube.

A model containing a frequency-independent resistance (R) and inertance (I) and a tissue damping (G) and elastance (H) of a constant-phase tissue compartment was fitted to the Z<sub>rs</sub> spectra by minimising the weighted difference between the measured and the modelled impedance data. The tissue parameters characterise the damping (resistive) and elastic properties of the respiratory system. Raw and law represent primarily the resistance and inertance of the airways, since the contribution of the chest wall to these parameters in rats is minor. The resistance and inertance of the ET tube and the connecting tubing (R<sub>ET</sub> = 125 cmH<sub>2</sub>O.s/l, I<sub>ET</sub> = 0.79 cmH<sub>2</sub>O.s<sup>2</sup>/l) were subtracted from the Raw and law values, respectively. Respiratory tissue hysteresivity ( $\eta$ ) was calculated as  $\eta = G/H$

### 3.3. BAL fluid collection and analyses

There was performed partial lung lavage in the animals. Positional changing of the examined animals provided that the washing fluid only can get into the left side lung. After the animal had been detached from the ventilator, it was held in an upright position and turned to the left. 0.8 ml pre-warmed (37 °C) physiological saline was instilled via a PE washing catheter (4-cm-long, 0.86-mm-ID and 1.27-mm-OD), which was led through the tracheal tube. To ensure that the washing liquid reached the lower airways, the animals were attached to the ventilator for 2 min. The rat was then detached from the ventilator again, its head was turned downwards and to the right, and the BAL fluid was collected gently from the tracheal cannula without suction. The presence of foamy air bubbles in the fluid indicated that the sample came from the bronchoalveolar space. Recovery of the washing fluid was 0.3-0.5 ml (40-60%). The BAL fluid was collected into Eppendorf deep freezing vessels, then it was centrifuged at 8000 rpm with an Eppendorf Biofuge Bico centrifuge. The supernatant was removed and the cell pellet was re-suspended in 50  $\mu$ l physiological saline. The total number of cells (TC) was counted with a haemocytometer (ABX Micros 60) and in a Buerker chamber. The cells were also smeared, fixed and stained with May-Grünwald-Giemsa and examined microscopically to identify the different cell types.

### 3.4. LPS treatment procedures

In 13 animals one week after the first measurements of respiratory mechanics and BAL, acute lung injury was induced by the ip injection of LPS (E. coli O55:B5, Sigma Chemical, St Louis, MO, USA) 18 h before the second measurement (ALI-group). Five rats received the same volume of physiological saline ip also 18 h before the second measurements (control group). The third measurements were made 2-4 weeks after the LPS or saline treatment (3 weeks on average). With this protocol, we had the possibility to compare the values for the two groups and also to follow the alterations in the same animals.

### 3.5. OVA treatment procedures and protocol groups

Four groups of Wistar rats were studied. After the first assessment of lung tissue mechanics included the responsiveness by performing iv MCh challenges and BALF collections, the rats were assigned into one or the other of the protocol groups as below.

The animals in Group 1 (N = 6) received an ip injection of 1 mg OVA and 50 mg

aluminium hydroxide (Sigma-Aldrich Ltd, Budapest, Hungary) on days 0 (after completing the first experiment) and 7. The experiments in these rats were performed on days 0 and 14. The rats in Groups 2 (N = 7) and 3 (N = 7) similarly received an ip injection of 1 mg OVA and 50 mg aluminium hydroxide on days 0 (after completing the first experiment) and 7. These rats were then exposed to aerosolised (Voyage Mefar jet nebuliser, Italy) OVA (25 mg/ml in saline, driven by a flow rate of 8 l/min of compressed air) during a 20-min period prior to the experiments. The second set of experiments was then performed on day 14 in the animals of Group 2, and on day 20 in the rats involved in Group 3.

The Group 4 (N = 10) rats received an ip injection of 1 mg OVA and 50 mg aluminium hydroxide on day 0 (after completing the first experiment). Aerosolized OVA was then administered to these animals on 7 consecutive days (days 14-20). Experiments were performed on day 0 and on day 20.

On the days of the experiments, when the animal had reached a steady-state condition (5-10 min after the starting of mechanical ventilation), the volume history was standardised by administering a hyperinflation via occlusion of the expiratory port of the ventilator. Four Zrs recordings were then collected to establish the baseline respiratory mechanical parameters. 2 mg/kg MCh was next administered into the tail vein, and Zrs was recorded 20 s, 1 min and 2 min after the injection. The preliminary experiments in another group of rats revealed that the peak response occurs 20 s after the MCh injection, whereas the effect was diminished 2 min later. Following this transient constriction, the animal was allowed to recover, and further iv MCh challenges were given by elevating the dose to 4, 8 and 16 mg/kg. The peak increases in the mechanical parameters were observed 20 s after the MCh injection; these increases were related to those obtained from the average of the 4 baseline Zrs recordings. After completion of the MCh provocations, partial lung lavage was performed.

### **3.6. Recovery management**

After the completion of the BAL and respiratory mechanical measurements, we waited for the return of spontaneous breathing, applied oxygen therapy according to the saturation change and finally extubated the animals.

### **3.7. Histological examinations**

For morphologic studies of the lung tissue the LPS-treated rats were euthanised with a 10% potassium chloride intravenous (iv) injection, following repeated BAL and FOT examination series. Among the chronic OVA treated rats (Group 4), 2 animals were lost after FOT and BAL measurements, in this way their lungs were examined histopathologically.

After opening the chest, the lungs and the heart were removed in one block, fixed by intratracheal inflation with 10% buffered formalin. Then we placed them into formalin for 48 hours. Representative lung tissue sections (from the mid portion of both lungs) were embedded in paraffin before cutting. The slides were stained with haematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Masson's trichrome. All slides were examined by the same investigator in a blinded fashion. A lung injury score was used to quantify the changes in lung architecture observed on light microscopy.

#### **3.7.1. LPS-treated animals**

The degree of microscopic injury was scored with regard to the following variables: BALT tissue hyperplasia of the bronchial walls, alveolar and interstitial damage (i.e., diffuse alveolar damage – DAD, atelectasis, and emphysema) and vascular alterations (hyperaemia

characterised by hypoxemia, pulmonary infiltrates, increased microvascular permeability and endothelial barrier disruption. ALI is often induced in animal models by administration of LPS, which a characteristic component of the cell wall of Gram-negative bacteria. LPS administration results in endotoxaemia similar to that seen in ARDS. The ensuing inflammation is reflected in a dramatically increased level of neutrophils in the BAL fluid. Our finding of a 10-fold increase in total cell count confirmed the previously demonstrated presence of acute inflammation 18 h after LPS administration. Furthermore, the present development of a noninvasive technique for BAL fluid collection allowed the follow-up of the BAL fluid profile weeks after the LPS injection. The cell count remained elevated, suggesting that LPS induced not only acute, but also long-lasting lung inflammation.

In agreement with the results of previous studies, the elevated cell count 18 h after the induction of experimental ALI was associated with significantly deteriorated parenchymal mechanics, while the airway parameters remained at their control levels. Unexpectedly however, despite the slight further elevations in total cell counts weeks after LPS exposure, the tissue mechanical parameters decreased significantly. A similar, apparently conflicting finding was observed in mice 48 h after the intranasal instillation of LPS, but the explanation of this observation is not completely clear. To explain the underlying mechanism responsible for this finding, the lungs in 3 LPS-treated rats were excised after completion of the protocol; histology systematically revealed the presence of emphysema, which may develop after LPS administration and leads to decreases in G and H.

- We have also utilised a method that allows follow-up of the BALF cell counts and airway and tissue mechanics in rats to assess the effects of different OVA treatment protocols on the lung responsiveness and on the inflammatory markers within the same rat.
- There were established, that repeated inhalations of the OVA after an ip injection of the allergen induced an influx of inflammatory cells (mainly eosinophils) into the bronchoalveolar system, which was associated with bronchial hyperreactivity to a non-specific constrictor agonist, MCh.

Effects of systemic OVA treatments are expected to cause mild-to-moderate alterations in the lung tissue. Accordingly, the longitudinal studies, particularly the follow-up of BALF and respiratory mechanics ensure detections of changes in individual animals and avoid the inter-individual variability. There have been only a few previous studies in monkeys and rats where individual animals served as their own controls. The behavior of small rodents (mice and rats) has not been fully characterised in this regard, despite the fact that these species are the most common animal models to study the pathogenesis of allergic lung diseases, such as asthma. In agreement with previous results, we did not observe any significant effects of the OVA treatments on the baseline values of the respiratory mechanical variables. The results we obtained in Group 1 were also in line with previous observations, confirming that ip administration of the allergen alone does not lead to the infiltration of inflammatory cells into the BALF and has no effect on the airway responsiveness. The earlier results relating to the effects of inhaled allergen are far more controversial. Some authors reported no effects of a single inhalation of OVA on the lung responsiveness, while others provided evidence of the development of BHR and lung inflammation following a single exposure to an aerosol of various allergens. The similarity between the experimental results obtained in Groups 2 and 4 demonstrates that time effects



- Our results confirmed the presence of a substantial inter-individual variability in cell count and respiratory mechanics under baseline conditions in individual rats.

Most studies in small rodents that required measurements of the BAL fluid and/or pulmonary function could be performed only once on the same animal. The ability to perform longitudinal studies has many obvious advantages. Accordingly, efforts have been made to develop techniques, which allow repeated intubation, the sampling of airway inflammatory cells and longitudinal assessment of the lung function in individual small rodents. Two different approaches have been proposed to collect lavage fluid repeatedly in small laboratory animals. Walters et al. developed procedures that allow individual mice to survive a whole-lung lavage. However, the washing procedure mandatory to obtain a sufficient amount of whole-lung lavage fluid in mice was likely to lead to a complete loss of surfactant function in their study. This was reflected in the lasting (1-4 days) deterioration in lung resistance and compliance and in variable increases in the number of inflammatory cells. Alternatively, segmental BAL provides information analogous to that from whole-lung lavage. Moreover, this technique is less harmful for the animal and it can be performed in larger rodents, such as rats or guinea pigs. In this regard, the paper by Varner et al. provides the only report of a method of segmental BAL that can be performed repeatedly in rats up to now. In their study, a small catheter was passed through a tracheal tube and was advanced to a wedge position. 0.1 ml of warm sterile buffer solution was instilled and then withdrawn by gentle suction and this maneuver was performed repeatedly. The results illustrated that the results of cell population measurements during acute inflammation differed considerably between samples obtained via segmental BAL versus total-lung lavage. The lavages were conducted in 2-week intervals, when changes in cellular profile or pulmonary resistance from the baseline were not observed.

Our study differs in many important aspects from all earlier ones. First, unlike the mice in the procedure developed by Walters, the rats were not ventilated with 100% O<sub>2</sub> in the present work, because we intended to test our method by using LPS, and hyperbaric O<sub>2</sub> might have reduced the ALI caused by the intratracheal spraying of LPS in rats. Furthermore, we did not use suction to withdraw the washing fluid from the bronchoalveolar cavity: it was collected only via a positional change of the animal. This method is sufficient to obtain around 50% of the instilled solution, it is believed to be more physiological and it causes less bleeding complications. The cell number and cell population in the BAL fluid did not differ significantly between samples collected by total-lung lavage or by our own method.

- It has been detected the longitudinal changes within the same rat in BALF cellular profile and lung tissue mechanics by induction an ALI/ARDS with ip injection of LPS.
- Another methodological improvement in the present study is that the effects of ALI induced by LPS were also detected, both in changes in cell counts and in the lung tissue mechanics. This technique can be used to estimate short- and long-term changes in airway mechanics and is suitable for studying the progression or improvement of a disease in a relatively small group.

ALI or ARDS, a major cause of morbidity and mortality in intensive care units, is

and haemorrhage). The severity of injury was graded as follows for each of the 4 variables: no injury (0), injury to up to one third of the field (1+), injury to up to two thirds of the field (2+), and diffuse injury throughout the field (3+).

### 3.7.2. OVA-treated animals

Characterisation of lung tissue inflammation including fields around bronchus/bronchiolus walls and vessels was performed by a semiquantitative score.

The severity of inflammation was graded on the basis of numbers and quality of inflammatory cells as follows for each of 3 variables: no injury (0), mild alteration (1+); moderate alterations (2+).

## 3.8. Statistical analysis

### 3.8.1. LPS - treated animals

The scatters in the parameters were expressed as SE values. The Kolmogorov-Smirnov test was used to test data for normality. Within the protocol groups, repeated measures of one-way analysis of variances (ANOVA) was used to assess the effects of time on the mechanical parameters and on the total cell counts. One-way ANOVA was applied to compare the mechanical parameters between the independent protocol groups. The Student-Newman-Keuls multiple comparison procedure based on the means was applied to compare the different conditions (for repeated measures) or protocol groups (for independent groups). Statistical tests were performed with a significance level of  $p < 0.05$ .

### 3.8.2. OVA - treated animals

The MCh dose causing a 100% increase in Raw (PC<sub>100</sub>) was calculated by linear interpolation of the dose-response curves from the individual animals. The scatters in the parameters were expressed as SE values. The Kolmogorov-Smirnov test was used to test data for normality. Two-way repeated measures of analysis of variances (ANOVA) with the factors assessment time (control vs. OVA treated) and group allocation was used to assess the effects of OVA treatments on the lung responsiveness and on the cell counts determined from the BALF. The Holm-Sidak multiple comparison procedure was applied to compare the different conditions (for repeated measures) or protocol groups (for independent groups). Correlation analyses between the variables were performed by using Pearson correlation tests. Statistical tests were carried out with the SigmaStat statistical software package (Systat Software, Inc, CA, USA) with a significance level of  $p < 0.05$ .

## 4. RESULTS

### 4.1. Analysis of BAL fluid and respiratory mechanics

Individual animal model was improved, which allows follow-up of BAL fluid cellular profile as well as the airway and tissue mechanics by methods of reproducible, partial BALF collections and separately measured airways and lung tissue mechanics with low frequency forced oscillation technique (FOT) in rats.

Although BAL led to a moderate, but statistically significant increase in Raw, the airway mechanics normalized within 3 min following the BAL procedure. The increases in the respiratory tissue parameters were more pronounced and lasted longer than those in Raw, but both G and H had returned to their baseline values 6 min after BAL. The total cell count was low and remained stable throughout the study period in the control animals suggesting

that the lavage procedure itself did not cause any significant change in the cell counts. The qualitative cell analysis of the BALF showed that, the ratio of macrophages was 50-60% of the TC.

#### **4.1.1. LPS-treated animals**

Longitudinal changes were identified within the same rat in BALF cellular profile and lung tissue mechanics by induction a mild ALI with an intraperitoneal injection of LPS.

LPS administration before the second set of measurements had minor effects on the parameters *Raw* and *Iaw*, whereas statistically significant increases were observed in both *G* and *H* at week 2. The mechanical parameters were fairly reproducible; they exhibited only slight gradual decreases in time in the control animals, which is probably due to lung growth during the experimental period. The elevations in *G* and *H* had diminished by the third set of measurements, i.e. 2-4 weeks after LPS administration. The mechanical parameters did not reveal any statistically significant difference between the protocol groups at any time during the experiment, while the statistical tests performed within a protocol group were sensitive to detect the LPS-induced changes. The TC rose markedly and significantly (with substantial inter-individual variability) after the second experiment in the LPS-treated rats.

To further characterise the effects of LPS on the lungs, we expressed the relative changes in the airway and tissue parameters between the first and the second week of the experiments in both groups of rats. The mild decreases in the airway mechanical parameters were associated with moderate increases in *G* and *H* following LPS administration, while the latter parameters remained at their pre-existing levels in the untreated rats. LPS induced striking elevations in TC, whereas TC was low and highly reproducible in the untreated rats.

Cytological patterns of BAL fluids showed marked differences between control and LPS-treated animals, furthermore the 2 and 3 mg/kg LPS-treated animals.

#### **4.1.2. OVA-treated animals**

By methods of serial measurement of BALF cellular profile and lung tissue mechanics in individual rats, bronchial hyperreactivity and the influx of cells into the lungs were assessed after they had been treated different modes of administration of the allergen, OVA.

There was no difference between the protocol groups in the baseline levels of the respiratory mechanical parameters, and none of the OVA treatment protocols affected their levels significantly. MCh induced dose-dependent increases in *Raw* and *G* under all experimental conditions, while *H* remained at the baseline level. OVA treatments had no significant effects on the MCh dose-response curves in Groups 1, 2 and 3, whereas the elevations in *Raw* were statistically greater ( $p < 0.05$ ) after sensitisation with chronic OVA exposure in the Group 4 rats. MCh induced dose-dependent increases in *Raw* in all protocol groups under both control and treated conditions ( $p < 0.001$  for all groups). No change in airway responsiveness was observed in the rats in Group 1 ( $p = 0.23$ ), Group 2 ( $p = 0.35$ ) or Group 3 ( $p = 0.51$ ) after treatment with OVA. In contrast, the MCh-induced changes in *Raw* were significantly enhanced ( $p < 0.02$ ) following the sensitisation procedure in the animals in Group 4.

The cellular content of the BAL exhibited a great inter-individual variability (ranging from  $6 \times 10^4$  to  $21 \times 10^4$  cells), though the group means of the initial values were not statistically different ( $p = 0.9$  for total and  $p = 0.7$  for eosinophil counts). OVA treatment did not cause any significant change in the total or eosinophil cell count in the rats in Groups 1, 2 and 3, whereas it led to marked and statistically significant increases in the total number of cells

( $p < 0.05$ ) and in the amount of eosinophils ( $p < 0.002$ ) in the animals in Group 4. No statistically significant changes were observed in the number of neutrophils.

Associations between the individual changes in the MCh dose causing 100% increase in the airway resistance ( $PC_{100}$ ) and the eosinophil content of the BALF in the rats Group 4 showed that decreases in  $PC_{100}$  following multiple OVA exposures were always associated with increases in the number of eosinophils in the BALF in the rats in Group 4.

#### **4.2. Histology**

Histopathological consequences of lung tissue were confirmed followed by LPS and OVA exposures in rats.

##### **4.2.1. LPS-treated animals**

Correspondingly with the cytological examinations, there are striking differences between the control and the LPS-treated lung tissue sections. Round cell infiltration was more expressed in the lung tissue of the rats treated with 3 mg/kg as compared with those treated with 2 mg/kg LPS. In the rats treated with 3 mg/kg LPS, severe pathological changes could be detected: appearance of lymphoid hyperplasia (partly follicular, partly parafollicular in the thickened bronchus walls) and bronchial intraepithelial lymphocytosis. The follicular hyperplasia was mild and not atypical, with the appearance of large number of plasmocytes. On the other hand, in the rats treated with 2 mg/kg LPS, mild expression of lymphocytes, plasmocytes macrophages, and mastocytes was observed. Control rats exhibited no pathological changes.

In the LPS group, 10 of the 13 animals had BALT hyperplasia. Hyperaemia was observed in 12 cases as a consequence of perivascular inflammation. Pathological signs of ARDS, DAD (i.e., oedema, alveolar hyperaemia, haemorrhage, pneumocyte II alterations, alveolar macrophage accumulation, and the development of hyaline membrane) and emphysema, were observed in 5 and 8 cases, respectively. In control rats, there were no pathologic changes in the bronchus wall or in the pulmonary interstitium, only mild hyperaemia was observed in 2 animals.

##### **4.2.2. OVA-treated animals**

Examinations of lung tissue specimens from OVA-treated rats demonstrated not only increased peribronchial/peribronchiolar but perivascular cellular infiltrates consisting of neutrophils, lymphocytes and eosinophils. Distributions of inflammatory cells were the similar between lymphocytes, plasma cells and eosinophils. There was no basal membrane thickening and or mucous exsudate in the bronchial lumen. In contrast to the OVA treated animals, the histological examinations of saline-treated control animals showed normal lung structure.

## **5. DISCUSSION**

It has been developed a novel approach that allows repeatable measurements of the BAL fluid cellular profile and the airway and respiratory tissue mechanics in individual rats.

- The present study has been demonstrated that reproducible BAL fluid and respiratory mechanical parameters can be obtained within the same rat.
- Our data support that collection of BAL fluid induced only a short-term deterioration in the respiratory function.