

Repetitive measurements of pulmonary mechanics to inhaled cholinergic challenge in spontaneously breathing mice

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Glaab, Thomas, Wayne Mitzner, Armin Braun, Heinrich Ernst, Regina Korolewitz, Jens M. Hohlfeld, Norbert Krug, and Heinz G. Hoymann. Repetitive measurements of pulmonary mechanics to inhaled cholinergic challenge in spontaneously breathing mice. *J Appl Physiol* 97: 1104–1111, 2004. First published April 30, 2004; 10.1152/jappphysiol.01182.2003.—Precise and repeatable measurements of pulmonary function in intact mice are becoming increasingly important for experimental investigations on various respiratory disorders including asthma. Here, we present validation of a novel in vivo method that, for the first time, combines direct and repetitive recordings of standard pulmonary mechanics with cholinergic aerosol challenges in anesthetized, orotracheally intubated, spontaneously breathing mice. We demonstrate that, in several groups of nonsensitized BALB/c mice, dose-related increases in pulmonary resistance and dynamic compliance to aerosolized methacholine are reproducible over short and extended intervals without causing detectable cytological alterations in the bronchoalveolar lavage or relevant histological changes in the proximal trachea and larynx regardless of the number of orotracheal intubations. Moreover, as further validation, we confirm that allergic mice, sensitized and challenged with *Aspergillus fumigatus*, were significantly more responsive to cholinergic challenge ($P < 0.01$) and exhibited marked eosinophilia and lymphocytosis in bronchoalveolar lavage fluids as well as significant pathological alterations in laryngotracheal histology compared with nonsensitized mice. We suggest that this approach will provide useful and necessary information on pulmonary mechanics in studies of various respiratory disorders in mice, including experimental models of asthma and chronic obstructive pulmonary disorder, investigations of pulmonary pharmacology, or more general investigations of the genetic determinants of lung function.

experimental animal models; pulmonary function test; allergy; bronchial asthma

THE ABILITY TO MEASURE PULMONARY FUNCTION in individual mice on repeated occasions is of great interest because of the prominent role played by these animals in genetic and translational research into the causes and mechanisms of respiratory and allergic diseases such as asthma (5, 14). It is becoming increasingly evident that the real potential of gene- or cell-based applications in experimental asthma models is tightly linked to a solid understanding of the underlying respiratory pathophysiology (20). This growing interest in functional studies with mice that correlate in vitro findings with in vivo determination of respiratory physiology has prompted the

search for measurements of pulmonary function that are valid and easy to implement.

Existing methods for measuring respiratory function in mice in vivo include noninvasive and invasive technologies (2, 5). Noninvasive determination of airway responsiveness (AR) in intact conscious mice has gained much interdisciplinary interest as a convenient and effective method for screening respiratory function over extended periods of time (11).

Concerns with existing noninvasive methods in spontaneously breathing mice include the contribution of upper pulmonary resistance (RL) and the uncertainty about the exact magnitude of bronchoconstriction (5). In particular, the utility and validity of the widely used enhanced pause (Penh) method (10) as a meaningful and physiological measure of bronchoconstriction have been seriously questioned recently by several authors (1, 15, 17, 19). The invasive determination of RL and dynamic compliance (C_{dyn}) after a bronchoconstrictor challenge is the gold-standard parameter measured in AR studies with mice (5, 16). To date, the utility of such conventional RL and C_{dyn} measurements are limited by invasive procedures such as tracheostomy, which precludes the practicality of repeated measurements and the need for anesthesia or mechanical ventilation. The ability to make repeated measurements of classical pulmonary mechanics is essential in long-term follow-up studies on the same animal. Moreover, it seems desirable for bronchial provocation test in mice to replicate the test conditions for diagnosing airway hyperresponsiveness in humans using inhaled cholinergic challenges. Unfortunately, a method that combines repeatable determinations of standard pulmonary mechanics in conjunction with local aerosol administration via an orotracheal tube in intact, spontaneously breathing mice is still lacking.

The purpose of the present study was to develop a simple approach that enables repetitive evaluation of pulmonary mechanics to aerosol challenges in orotracheally intubated, spontaneously breathing mice. To this end, we first identified whether pulmonary function tests (PFT) in response to aerosolized methacholine (MCh) were reproducible over short and extended periods of time and, second, evaluated whether we could differentiate between levels of AR in nonsensitized and allergic mice sensitized to *Aspergillus fumigatus*. Next, we examined the effects of repeated PFT to inhaled MCh on laryngotracheal histology and cytological parameters in bronchoalveolar lavage (BAL) fluid. The adaptations that we have

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made to the technique should permit accurate, routine, and repeatable analysis of murine respiratory mechanics in individual mice.

MATERIALS AND METHODS

Animals and sensitization protocol. Pathogen-free, female BALB/c mice (Charles River, Sulzfeld, Germany), 12–14 wk of age at the time of the first PFT or BAL, respectively, were kept in a pathogen-free rodent facility and were provided food and water ad libitum. All animal experiments conformed to the guidelines of the National Research Council Guide and were approved by the appropriate governmental authority (Bezirksregierung Niedersachsen, Germany). Mice were separated into groups ($n = 8$ each) defined by the sensitization and measurement protocols (Table 1). Allergic mice received an intraperitoneal and subcutaneous injection of soluble *A. fumigatus* antigens (2.7 μg each, Greer Laboratories, Lenoir, NC) dissolved in incomplete Freund's adjuvant in a volume of 0.1 ml given on *day 0* and were challenged noninvasively in a closed chamber with 1% of *A. fumigatus* aerosol dissolved in saline for 12 min on *days 14* and *21* (Pari Master, LC Star, 2.8- μm mass median aerodynamic diameter, Pari, Starnberg, Germany). On *day 23*, allergic mice were challenged once with aerosolized MCh as described below. The other six groups of nonsensitized mice varied in the total number of PFT measurements to MCh challenge (ranging from 0 to 6) and the time intervals between PFT recordings (Table 1).

PFT to cholinergic challenge. AR was assessed as an increase in RL after challenge with aerosolized MCh (Sigma, Deisenhofen, Germany) in anesthetized, spontaneously breathing mice using a modification of the techniques described by Brown et al. (3). Briefly, mice were anesthetized with intraperitoneal injections of etomidate (total dose: 21.5–37.5 mg/kg) and fentanyl (total dose: 0.10–0.19 mg/kg) with minimal supplementations as required. When an appropriate depth of anesthesia, as monitored by a loss of the righting and pinch toe reflex, was achieved, mice were suspended by their upper incisors from a rubber band on a 65° incline Plexiglas support. The trachea was transilluminated below the vocal cords by a 100-W halogen light source with two 24-in. flexible fiber-optic arms to allow visualization of the trachea through the oral cavity. A modified mouse laryngoscope made from steel (length 12 cm plus an additional 1.8 cm at an angle of 135°, width 0.3 cm) was used as a suitable tool to provide a clear view of the tracheal opening. During this direct visualization, a standard 20 G \times 32 mm (1¼ in.) Abbocath T cannula (Abbott, Sligo, Ireland) was gently inserted into the tracheal opening. All mice included in the study were successfully intubated at the first attempt. The intubated, spontaneously breathing animal was then removed from the Plexiglas support and placed in supine position in a thermostat-controlled whole body plethysmograph as shown in Fig. 1 (designed by Fraunhofer ITEM in cooperation with HSE-Harvard Apparatus, March-Hugstetten, Germany). The orotracheal tube was directly

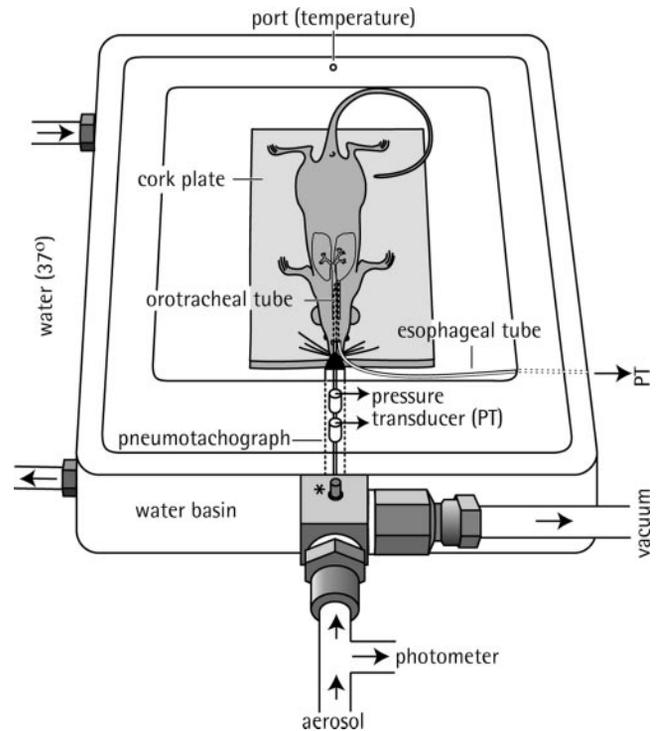


Fig. 1. Diagram of the plethysmograph used for pulmonary function testing of anesthetized, orotracheally intubated mice. A thermostat-controlled water basin (37°C) built in the plethysmograph chamber ensured a body temperature of 34–35°C as measured by a rectal thermometer. Defined aerosol concentrations of methacholine (MCh), as measured by an aerosol photometer, were delivered into the airways via the orotracheal tube. For the calculation of pulmonary resistance (RL), transpulmonary pressure (Ptp) was recorded via an esophageal tube, and tidal flow was determined by a pneumotachograph attached directly to the orotracheal tube. PT, pressure transducer. *Adaptor connected to a pressure control unit that maintained constant pressure conditions during measurements.

attached to a pneumotachograph (capillary tube PTM T16375; resistance: 0.136 cm H₂O · s · ml⁻¹; dead space: 24 μl ; flow linearity until 3.5 ml/s; HSE-Harvard) installed in the front part of the chamber. The resistance of the orotracheal tube (0.63 cmH₂O · s · ml⁻¹) was linear over the physiological range of flows studied (flow linearity until 3 ml/s). Tidal flow was determined by the pneumotachograph connected to a differential pressure transducer (Validyne DP 45-14, HSE-Harvard).

To measure transpulmonary pressure (Ptp), a water-filled PE-90 tubing was inserted into the esophagus to the level of the midthorax and coupled to a pressure transducer (model P75, HSE-Harvard) (24). The optimal positioning of the esophageal tube was in the lower one-third of the esophagus where we monitored maximum negative pressures. The frequency response of the esophageal catheter/pressure transducer unit was flat over the range of 1 to 23 Hz. The amplified analog signals from the pressure transducers were digitized by an analog-to-digital converter (DT 302, Data Translation, Marlboro, MA) at a sampling rate of 250 Hz. Before each experiment, calibrations of flow and pressure were performed with a volume of 0.2 ml of air and pressures of ± 8 and ± 16 cmH₂O, respectively. Potential air leaks around the orotracheal tube were assessed as previously described (3), and no functional leaks were found.

RL was determined from the ratio of Ptp to tidal flow over an entire breath cycle (8, 21). The resistance of the orotracheal tube (0.63 cmH₂O · s · ml⁻¹) was subtracted from all RL measurements. Cdyn was calculated as the quotient of tidal volume (VT) and Ptp from the end of the previous to the end of the present respiratory cycle. RL and Cdyn together with other basic respiratory parameters were continu-

Table 1. Definition of study groups

Group	Sensitization	PFT to Inhaled MCh	BAL and Histology
Control	–	none	<i>day 0</i>
PFT-1 \times	–	<i>day 0</i>	<i>day 1</i>
PFT-2 \times	–	<i>days 0, 7</i>	<i>day 8</i>
PFT-4 \times /wk	–	<i>days 0, 2, 4, 7</i>	<i>day 8</i>
PFT-4 \times	–	<i>days 0, 7, 14, 21</i>	<i>day 22</i>
PFT-6 \times	–	<i>days 0, 7, 14, 21, 28, 35</i>	<i>day 36</i>
PFT-allergic	+	<i>day 0</i>	<i>day 1</i>

Time schedule for pulmonary function testing (PFT) in several groups of nonsensitized and *Aspergillus fumigatus*-sensitized mice. PFT, bronchoalveolar lavage (BAL) and laryngotracheal histology were performed on the days indicated. $n = 8$ mice/group. Allergic animals at *day 0* had been sensitized by *A. fumigatus* as described in MATERIALS AND METHODS. MCh, methacholine.

ously recorded with commercial software (HEM 3.4, Notocord, Croissy, France). For each breath, a flow offset (Fref) and a pressure offset (Pref) were computed

$$F_{ref} = \frac{\sum_{t=T_{EndExp}}^{T_{EndExp}} F(t)}{T_{EndExp} - T_{EndExpPrev}}$$

$$P_{ref} = \frac{\sum_{t=T_{BegInsp}}^{T_{EndExp}} P(t)}{T_{BegInsp} - T_{EndExpPrev}}$$

where $F(t)$ is flow as a function of time, $P(t)$ is pressure as a function of time, $T_{EndExpPrev}$ is the end of expiration of the previous breath, $T_{BegInsp}$ is the beginning of inspiration, and T_{EndExp} is the end of expiration of the present breath. RL and Cdyn were averaged over a complete respiratory cycle using an integration method over flows, volumes (Vol), and pressures according to the following equations (21)

$$RL = \frac{\sum_{t=T_{EndExpPrev}}^{T_{EndExp}} [P(t) - Pref][F(t) - Fref]}{\sum_{t=T_{EndExpPrev}}^{T_{EndExp}} [F(t) - Fref][F(t) - Fref]}$$

$$C_{dyn} = \frac{\sum_{t=T_{EndExpPrev}}^{T_{EndExp}} Vol(t)Vol(t)}{\sum_{t=T_{EndExpPrev}}^{T_{EndExp}} Vol(t)[P(t) - Pref]}$$

Respiratory parameters were averaged in 5-s segments, and both maximum RL and minimum Cdyn values were taken and expressed as percent changes from corresponding baseline values. After the measurements, mice were removed from the chamber and extubated as soon as they began recovering from anesthesia.

Administration of MCh aerosols. After baseline values were recorded, AR to doubling doses of aerosolized MCh (0.125–0.25–0.5–1 μ g) in naive and in *A. fumigatus*-sensitized mice was assessed. Three to five minutes were allowed between doses to permit a return to or near baseline values. Dried aerosols were generated by a computer-controlled, jet-driven aerosol generator system (Bronchy III, particle size 2.1- μ m mass median aerodynamic diameter, Fraunhofer ITEM, licensed by Buxco, Troy, NY) as previously described for rats (8, 13). Aerosol concentrations were determined by a gravimetrically calibrated photometer. The total dose (0.125–1.0 μ g) inhaled via the orotracheal tube was calculated and controlled by a computerized dose-control system based on the continuously measured respiratory volume per minute and aerosol concentration. To prevent hypoxemia, the animals were provided with 40% oxygen during measurements including baseline.

BAL cell counts and laryngotracheal histology. Total and differential cell counts from BAL samples using 2 \times 0.8-ml aliquots of saline were determined as previously described (7), except that recovery of BAL fluids was performed from the distal trachea. After BAL, the proximal trachea and larynx were prepared and fixed in 10% neutral buffered formalin, trimmed, dehydrated, and embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin-phloxin. Histopathological evaluation of the larynx and trachea was performed by a pathologist blinded to treatment of the groups. Epithelial erosions and inflammation were scored using a subjective

semiquantitative scale of 0 (none), 1 (minimal), 2 (mild), 3 (moderate), and 4 (severe) as previously described (24).

Statistics. A mixed model with the animal as subject, group, time, and dose as fixed effects, and the animal as random factor was applied for global tests on group, dose, and time effects (4). Variables without repeated measurements were compared between experimental groups by Student's *t*-test (PFT) or Mann-Whitney test (cytology, histology). *P* values of <0.05 were considered significant. Descriptive results were expressed as means \pm SE unless indicated otherwise. Statistical analysis was performed with SPSS 11.5.

RESULTS

Baseline values of respiratory parameters. The initial baseline values (means \pm SD) for RL (0.94 \pm 0.24 cmH₂O·s·ml⁻¹),

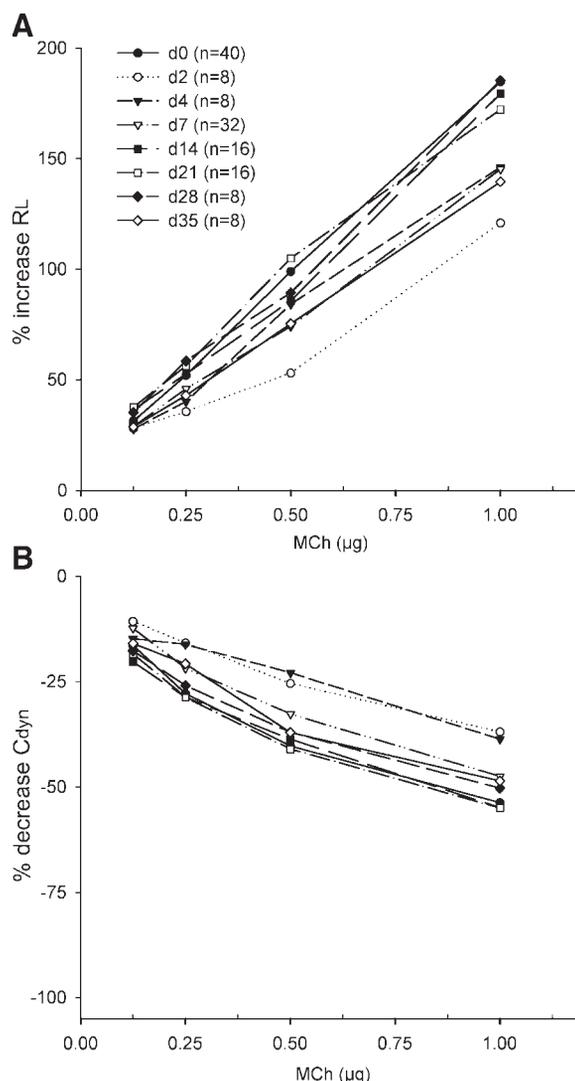


Fig. 2. Airway responsiveness (AR) of several groups of nonsensitized mice to inhaled MCh including pooled data from serial measurements of RL and dynamic compliance (Cdyn) from day (d) 0 through d35 (see also Table 1). A: dose-response curves were stable and demonstrated a small overall variability of peak RL values with increasing doses of inhaled MCh. There was a small but significant decrease in the magnitude of cholinergic AR after the second (d2 and d7, respectively) and sixth intubation (d35) ($P < 0.05$). B: dose-dependent decreases in Cdyn to MCh challenge were stable and demonstrated variations of peak Cdyn values especially in PFT-4 \times /wk mice (d2, d4). There were significant differences in the degree of cholinergic AR after intubations at d2, d4, and d7, respectively ($P < 0.01$). Values are means of 8–40 animals per group. PFT, pulmonary function test.

Cdyn (0.030 ± 0.004 ml/cmH₂O), V_T (0.14 ± 0.01 ml), and breathing frequency (f; 104 ± 10 breaths/min) of anesthetized, orotracheally intubated, spontaneously breathing BALB/c mice ($n = 40$) obtained during a 1-min control period were not significantly different from the baseline values of allergic animals ($n = 8$). To assess the variability and repeatability in individual animals, RL and Cdyn baseline values were measured without subsequent MCh exposures in another group of four naive mice four times once weekly. The individual coefficients of variation were 12, 9, 13, and 13% for Cdyn and 15, 16, 16, and 22% for RL, resulting in a mean coefficient of variation of 13% for Cdyn and 19% for RL.

Validation of repeated pulmonary function measurements to inhaled MCh challenge. To demonstrate that reliable measurements of RL and Cdyn to inhaled bronchoconstrictors can be repeatedly recorded in intact mice, dose-response studies to MCh aerosols were performed. Figure 2 illustrates that dose-dependent increases in RL and dose-related decreases in Cdyn to inhaled MCh were observed in all animals up to a total of 6 measurements and reveals small variabilities of resistance and compliance data during serial measurements among nonsensitized mice. The alterations in RL and Cdyn in response to MCh were paralleled by dose-related decreases in V_T {mean decrease \pm SE at the largest MCh concentration: $-23.7 \pm 1.1\%$ [95% confidence interval (CI): -21.5 to -26.6] for nonsensitized mice (pooled data of single and repeated recordings, $n = 104$) and $-57.5 \pm 2.7\%$ (95% CI: -51.2 to -63.9) for sensitized mice ($n = 8$)} as well as small increases in f at the largest MCh concentration [$13 \pm 1.3\%$ (95% CI: 10.4 to 15.6) for nonsensitized mice (pooled data, $n = 104$) and $6 \pm 6.9\%$ (95% CI: -10.4 to 22.4) for sensitized mice ($n = 8$)]. Figure 3 shows a characteristic example of the changes to RL, Cdyn, V_T, and f in response to cholinergic challenge.

To further examine whether there were any time or time-dose effects associated with repeated PFT recordings, we analyzed the magnitude of cholinergic AR among nonsensitized mice measured at different time intervals. Figure 4A

shows that no significant differences in cholinergic RL responsiveness were found between groups that were monitored four times either within a week (PFT-4 \times /wk) or once weekly (PFT-4 \times). In addition, no differences in AR were observed between PFT-4 \times /wk and animals that were measured twice a week. These data indicate that no time or time-dose effect was present and that RL measurements were stable at short and extended periods of time. In contrast, variations of Cdyn recordings with increasing doses of inhaled MCh in PFT-4 \times /wk mice resulted in significant time and time-dose effects between PFT-4 \times and PFT-4 \times /wk mice at the last two measurements ($P < 0.05$; Fig. 4B). Figure 5 shows that allergic mice sensitized and challenged with *A. fumigatus* were much more hyperresponsive for all doses of MCh exposure compared with nonsensitized animals measured at the same time point ($P < 0.01$).

Effects of single and repeated intubations on BAL cytology and on laryngotracheal histology. To study the influence of single and repeated PFT on airway inflammation and histological alterations of the proximal trachea and larynx, we investigated cytology from BAL samples and laryngotracheal histology. Table 2 shows that allergic mice elicited significant increases in eosinophils, lymphocytes, and, to a lesser extent, neutrophil counts ($P < 0.01$) compared with nonsensitized animals. Except for small variations in macrophages, no significant differences in BAL cell counts were observed among groups of nonsensitized groups at any time point.

Histological evaluation of the larynx and proximal trachea using a semiquantitative scoring system showed a significantly greater intensity of mucosal inflammatory cell infiltration in allergic mice (mean score: 2.4 ± 0.2) compared with naive mice ($P < 0.01$ except for PFT-1 \times and PFT-4 \times /wk: $P < 0.05$) (Figs. 6 and 7). The inflammatory infiltrate consisted of a mixture of granulocytes, with a high proportion of eosinophils in allergic mice, and mononuclear cells, predominantly comprising lymphocytes. As expected, histological alterations were completely absent in naive control mice. Inflammation scores

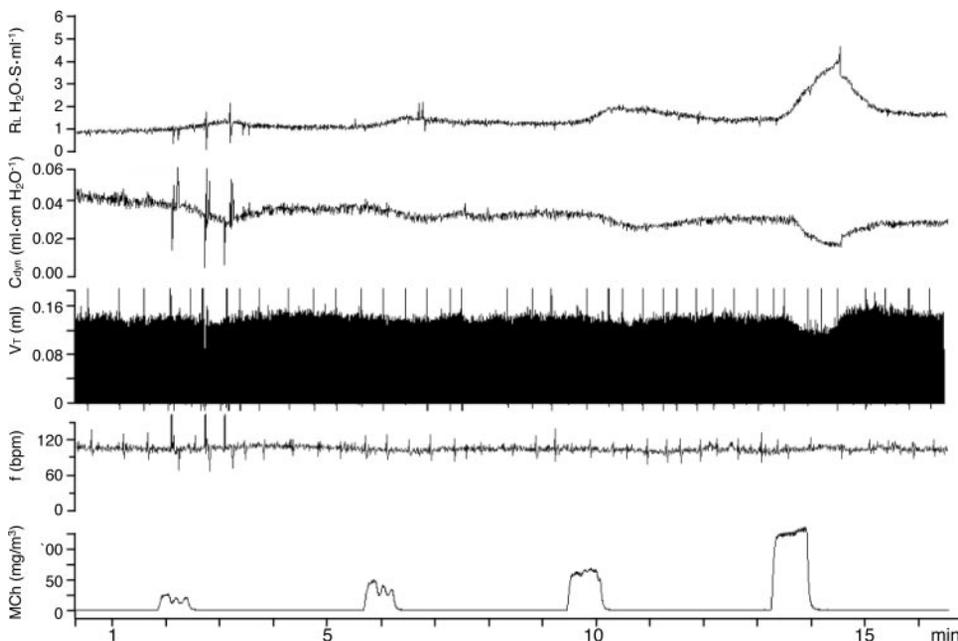


Fig. 3. Characteristic pattern of airway obstruction to increasing concentrations of aerosolized MCh, illustrating the changes in RL, Cdyn, tidal volume (V_T), and breathing frequency (f) in a nonsensitized, orotracheally intubated mouse. bpm, Breaths/min.

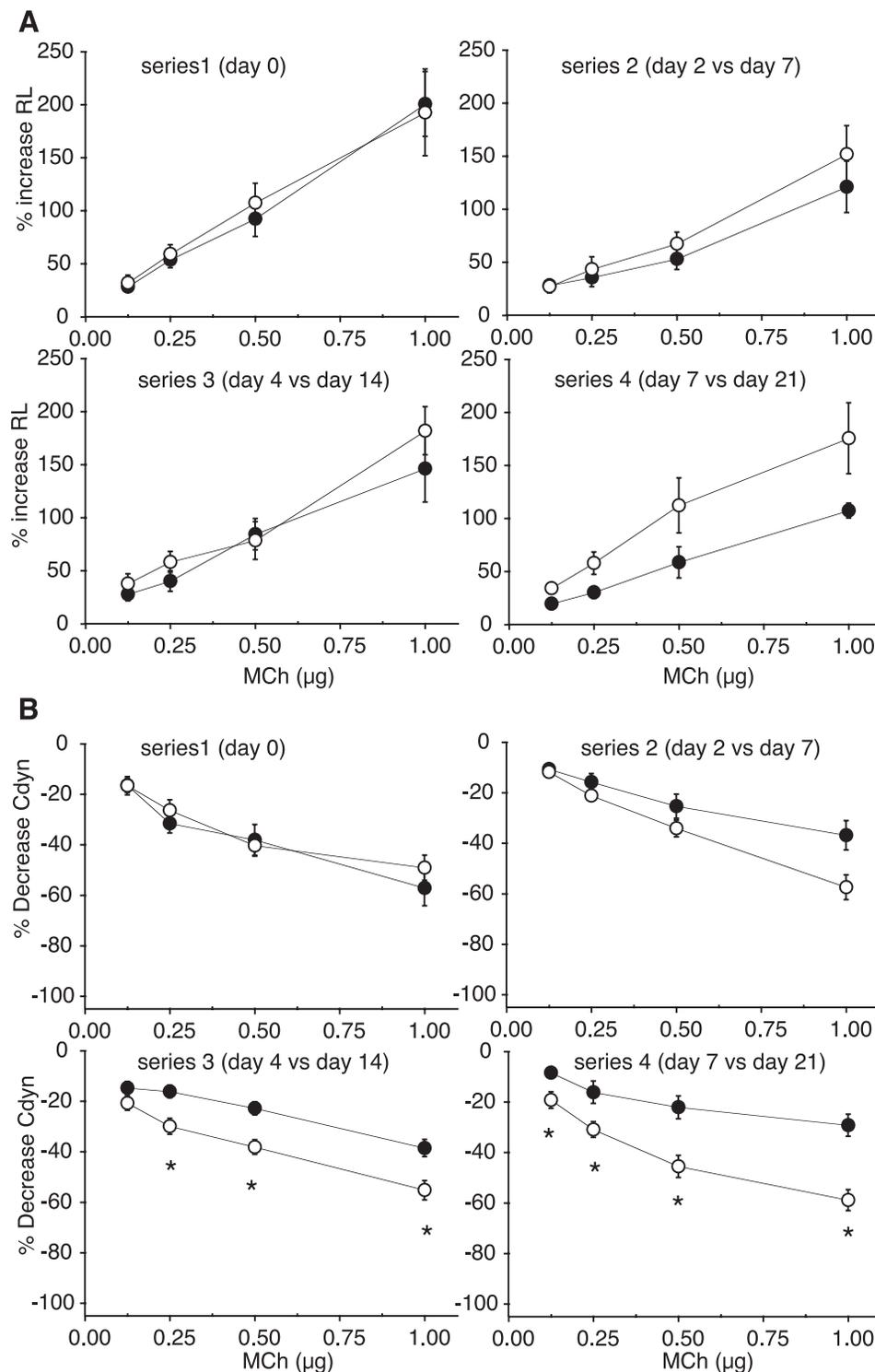


Fig. 4. Comparison of 4 dose-response curves to aerosolized MCh challenge in 2 groups of nonsensitized mice measured at different time intervals. PFT-4x/wk mice (●) were measured 4x times within 1 week. PFT-4x mice (○) were monitored 4 times once weekly. A: no significant differences in cholinergic AR, as measured by RL, were found between the 2 groups at each time point. B: in contrast, significant differences in AR as measured by Cdyn were observed between groups at sessions 3 and 4. Values are means ± SE (n = 8). *P < 0.05.

among nonsensitized groups with previous PFT ranged between minimal to mild without causing substantial intergroup differences except for PFT-4x/wk (mean score: 1.7 ± 0.2) vs. PFT-4x (mean score: 1.1 ± 0.2, P < 0.05).

Mean scores of epithelial erosions were significantly higher in allergic animals (PFT-allergic: mean score: 3.0 ± 0.5) compared with all nonsensitized mice (P < 0.01 except vs. PFT-1x: P < 0.05). Epithelial erosions were not associated with clinical signs of respiratory distress. It is noteworthy that

epithelial erosions were absent after four PFT measurements (PFT-4x/wk and PFT-4x). Variability in mean scores among the different groups of naive mice, ranging from none to minimal-mild (mean score range: 0–1.4 ± 0.6), resulted in significant differences (P < 0.05) between most of these groups, whereas nonsignificant changes were found between the groups control vs. PFT-4x/wk, control vs. PFT-4x, PFT-4x/wk vs. PFT-4x, and PFT-1x vs. PFT-6x. A variety of other small reactive pathological changes, such as squamous

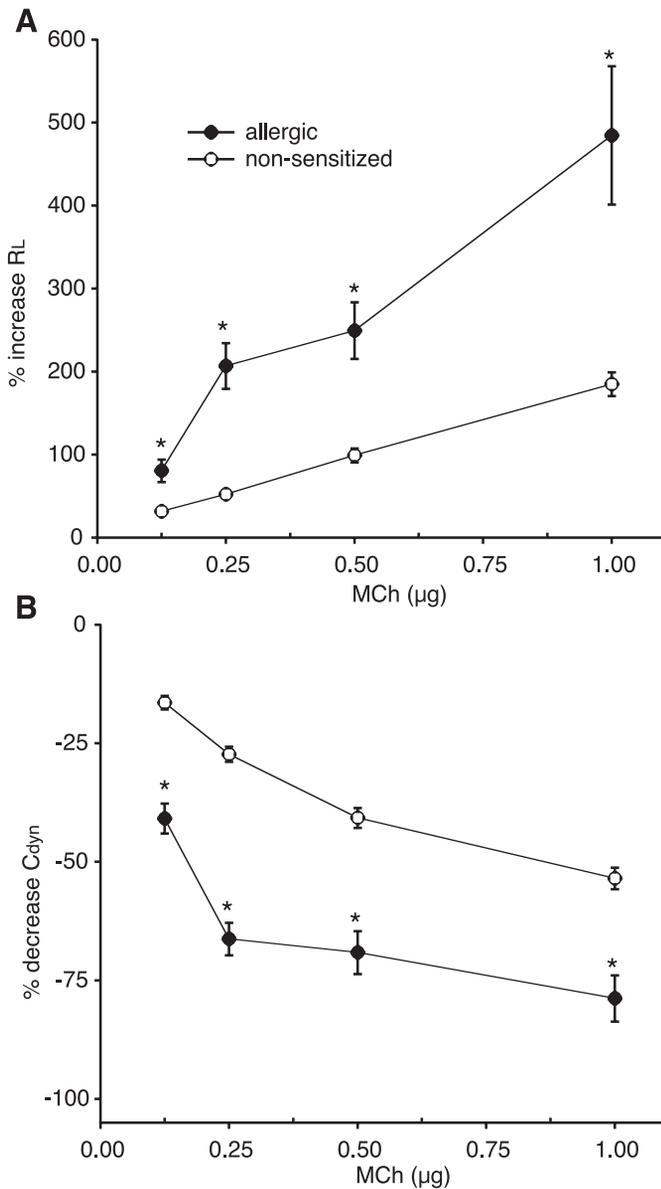


Fig. 5. AR to inhaled MCh challenge in orotracheally intubated, spontaneously breathing BALB/c mice. Dose-response curves to inhaled MCh showed significantly increased RL and decreased Cdyn values, respectively, for all doses of MCh and a shift to the left of the dose-response curve in allergic mice ($n = 8$) compared with nonsensitized animals at d0 ($n = 40$ pooled data). Values are means \pm SE. * $P < 0.01$.

cell metaplasia or respiratory epithelial atrophy, occasionally were observed in mice of different groups.

DISCUSSION

In the present study, we describe a novel approach that integrates repeatable measurements of standard mechanical lung parameters with direct aerosol provocation in orotracheally intubated, spontaneously breathing mice. Our results show that, in nonsensitized animals, airway responses to inhaled MCh challenge were stable over days and weeks without causing relevant airway inflammation or trauma. We were further able to discriminate the degrees of cholinergic AR, cellular influx in BAL fluids, and laryngotracheal pathologies between nonsensitized and allergic animals.

With the ability to manipulate the mouse genome in the context of the whole organism and the possibility of revealing the function of newly discovered genes in vivo (18), it is equally important to phenotypically assess these experimental models by accurate and repeatable measurements of airway mechanics in vivo. Many investigators in recent years have attempted to address this issue by utilizing whole body plethysmography, measuring an arbitrarily defined variable (Penh) derived from the shape of the pressure in the box (10). Although this approach represents the ultimate in simplicity, the measurement itself unfortunately has no theoretical link to airway size (15, 17) and, for this reason, often fails to correlate with more direct measurements of RL (1, 15, 19). Thus this plethysmographic approach fails to provide a meaningful solution to the problem. Introduction of the orotracheal intubation technique in mice by Brown et al. (3) has enabled monitoring of respiratory mechanics in intact individual mice, but the potential of that approach remains to be fully evaluated yet. The present study differs in two important ways from that initial report: first, because we have developed and applied a method that uses aerosolized MCh challenge, and second, because we make continuous measurements in spontaneously breathing mice. These modifications meet the demands for proper phenotyping because it is now possible to perform repetitive recordings of well-accepted mechanical lung parameters on the same animal without surgical interventions and mechanical ventilation. The baseline values for basic respiratory parameters in anesthetized, orotracheally intubated, spontaneously breathing mice were stable and similar to those reported previously for anesthetized, mechanically ventilated BALB/c mice (23).

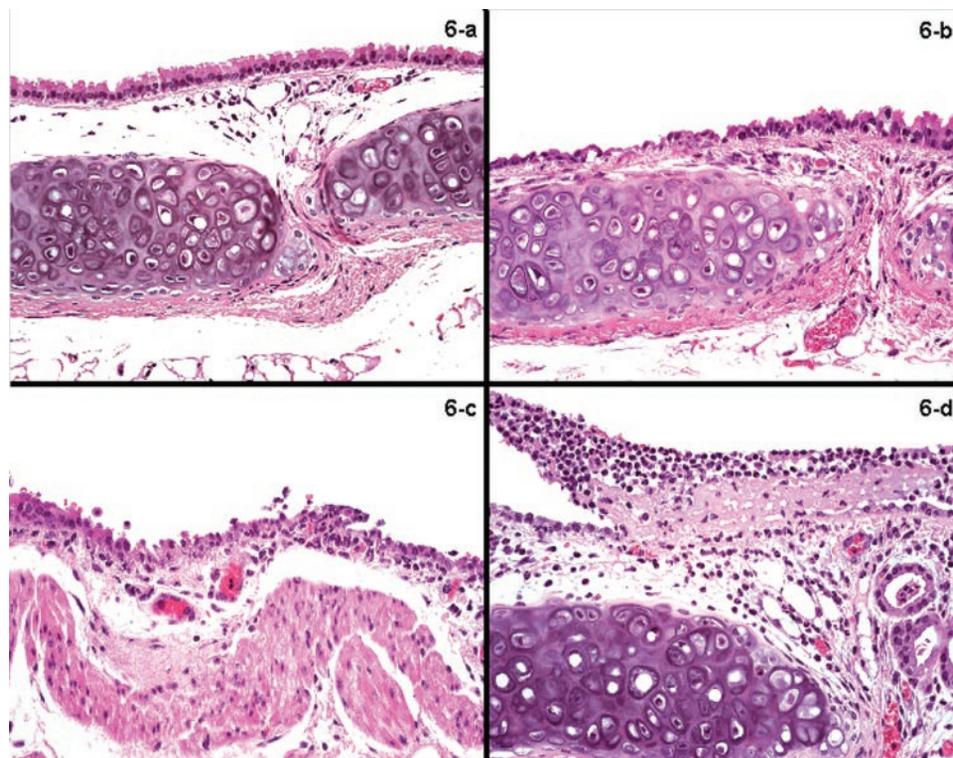
To evaluate the validity of this approach, we performed single and repetitive dose-response studies to aerosolized MCh followed by cytological and histological analyses of the airways in separate groups of intact mice (Table 1). Cholinergic aerosol challenges resulted in dose-dependent increases in RL and decreases in Cdyn over baseline values in all investigated animals. Importantly, the results of serial cholinergic challenges were stable and similar among nonsensitized animals. Despite there being small intrastrain variability in AR to MCh challenge consistent with other reports (24, 28), no evidence of tolerance or potentiation of cholinergic RL and Cdyn responsiveness was present with repeated measurements for as long

Table 2. Cellular composition of BAL fluid

Group	Eosinophils, $\times 10^4$	Neutrophils, $\times 10^4$	Lymphocytes, $\times 10^4$	Macrophages, $\times 10^4$
Control	<1	<1	<1	12.4 \pm 1.9
PFT-1 \times	<1	<1	<1	10.6 \pm 1.6
PFT-2 \times	<1	<1	<1	8.6 \pm 0.7
PFT-4 \times /wk	<1	<1	<1	8.9 \pm 1.8
PFT-4 \times	<1	<1	<1	10.3 \pm 1.2
PFT-6 \times	<1	<1	<1	7.1 \pm 0.6
PFT-allergic	36.1 \pm 6.6*	1.1 \pm 0.3*	13.5 \pm 2.7*	16.4 \pm 2.8

Values are means \pm SE; $n = 8$ mice/group. Leucocytes were recovered from BAL fluid according to Table 1. *Significant differences in the number of eosinophils, neutrophils, and lymphocytes were found between allergic and nonsensitized animals ($P < 0.01$). Small but significant differences ($P < 0.05$) in macrophage counts were found between the groups PFT-6 \times vs. control, vs. PFT-1 \times , vs. PFT-4 \times , vs. PFT-allergic and also between PFT-allergic vs. PFT-4 \times /wk and vs. PFT-2 \times .

Fig. 6. Spectrum of pathological changes in the proximal trachea. *a*: Unaltered respiratory mucosa (control group). *b*: Mild (*grade 2*) focal atrophy of the respiratory epithelium. The ciliated respiratory epithelium is focally replaced by less-differentiated, non-ciliated low cuboidal epithelial cells (PFT-4×). *c*: Moderate (*grade 3*) mixed inflammatory cell infiltration of the respiratory epithelium and underlying lamina propria (PFT-6×). *d*: Severe (*grade 4*) focal epithelial erosion and inflammation. Focal necrosis and granulocytic inflammation has focally effaced the respiratory epithelium of the trachea. The affected area is covered by a fibrinous exudate containing numerous granulocytes (PFT-allergic). Hematoxylin and eosinophilin, ×400.



as six measurements (Fig. 2). Comparison of serial RL measurements to aerosolized MCh further revealed no differences in the degree of AR between groups of nonsensitized mice measured several times, either within a week or once weekly, suggesting that no time or time-dose effect was present with repeated measurements of RL (Fig. 4A). The observation that *A. fumigatus*-treated mice were significantly more responsive to MCh challenge indicates that this method reliably differen-

tiates the degree of AR between allergic and nonsensitized animals (Fig. 5). Variations in airway responses to MCh were unlikely to have been due to variations in *f*, because we generally observed no relevant alterations in respiratory rate with MCh challenges. These results further underline the usefulness of computer-controlled aerosol generation that permits simple and accurate delivery of a well-defined dried aerosol through the orotracheal tube, thus enhancing aerosol deposition in the peripheral airways. Alternatively, ultrasonic nebulization of bronchoconstrictor solutions (mg/ml) has been successfully applied in tracheostomized, ventilated mice (22). Such a direct cholinergic challenge to the airways is advantageous over systemic challenge not only because it better simulates physiological stimulation of airway smooth muscle in humans but also because systemic delivery of agonists has been reported to affect airways via reflex or humoral mechanisms (6, 9).

Orotracheal intubation also offers the opportunity to perform BAL samples *in vivo* on multiple occasions in the same animals (27). This means that, in parallel to performing serial PFT, the full spectrum of inflammatory cells and its released mediators can be evaluated in longitudinal studies. The method can also be combined with alternative mechanical ventilation technologies that can separate airway from tissue resistance (23, 26). However, it should be recognized that, over time, mechanical ventilation might possibly contribute to lung injury, which may alter the nature of chronic inflammatory changes in murine models of asthma or chronic obstructive pulmonary disorder (25). Drawbacks of our technique include the need for anesthesia, nonsurgical instrumentation of the trachea, and technical limits in measuring several mice simultaneously. The general limitations on the measurement of

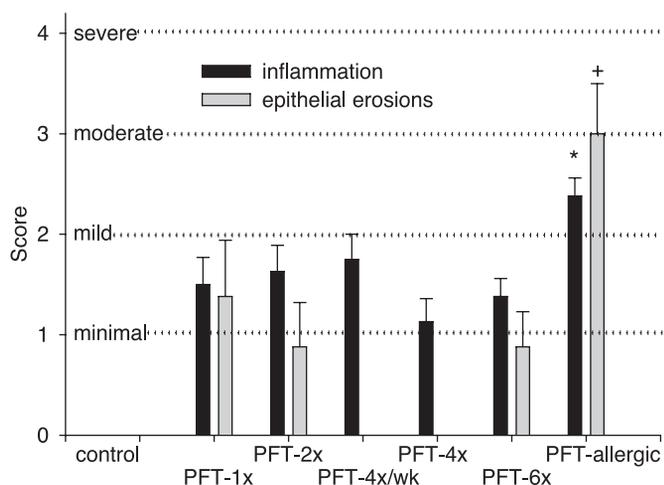


Fig. 7. Histological scoring scale for inflammation and epithelial erosions of the proximal trachea and larynx: 0 (none), 1 (minimal), 2 (mild), 3 (moderate), 4 (severe). Nonsensitized mice exhibited minimal to mild focal mucosal inflammatory cell infiltration and none (PFT-4×/wk; PFT-4×) to mild focal epithelial erosions, whereas the mean scores for inflammation and epithelial erosions were significantly higher in allergic mice. Values are means ± SE; *n* = 8 mice/group. **P* < 0.01: PFT-allergic vs. nonsensitized groups except vs. both PFT-1× and PFT-4×/wk (*P* < 0.05). +*P* < 0.01: PFT-allergic vs. nonsensitized groups except vs. PFT-1× (*P* < 0.05).

pulmonary mechanics in mice have been extensively reviewed recently (2).

One important issue of this report was to address the possibility that epithelial trauma due to orotracheal intubation per se might affect both pulmonary function and airway inflammation. An important finding of this study, however, was that repeated PFT recordings had no impact on leukocyte distributions in BAL samples among all groups of nonsensitized mice. This enabled us to show that airway hyperresponsiveness in *A. fumigatus*-treated mice was linked with pronounced eosinophilia and lymphocytosis in BAL (Table 2) (12). These results with BAL were supported by histopathological evaluation demonstrating that single and repeated PFT elicited only minor histological alterations of the proximal trachea and larynx in nonsensitized mice regardless of the number of PFT (Fig. 7). They are also consistent with previous work, which examined the effect of repeated BAL with orotracheal intubation (27). The fact that histological and cytological alterations were significantly more pronounced in allergic mice cannot be attributed to single orotracheal intubation but rather reflects enhanced inflammation and vulnerability of the conducting airways in allergic mice.

In summary, our results describe a new method that enables reproducible follow-up studies of well-accepted pulmonary mechanical parameters to defined cholinergic aerosol challenge in orotracheally intubated, spontaneously breathing mice without causing relevant laryngotracheal trauma or airway inflammation. We suggest that this approach will provide useful and necessary information on airway and parenchymal mechanics in studies of various respiratory disorders in mice, including experimental models of asthma and chronic obstructive pulmonary disorder, investigations of pulmonary pharmacology, or more general investigations of the genetic determinants of lung function.

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