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toolbox

Assessment of cellular profile and lung function with repeated bronchoalveolar lavage in individual mice

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Walters, Dianne M., Marsha Wills-Karp, and Wayne Mitzner. Assessment of cellular profile and lung function with repeated bronchoalveolar lavage in individual mice. *Physiol. Genomics* 2: 29–36, 2000.—In this study, we sought to develop procedures that would enable repeated bronchoalveolar lavage (BAL) in individual mice on multiple occasions. To achieve this objective, we first developed the procedures that would allow individual mice to survive a whole lung lavage, and then tested whether, on subsequent days, there was an effect of this initial BAL on the cell profile, lung permeability, and baseline respiratory function. Our results demonstrate that the repeated lavage procedure can be readily carried out in individual mice of different strains on multiple occasions. The lavage procedure itself results in immediate increases in respiratory system resistance and concomitant decreases in compliance, but these parameters return to prelavage values by the 2nd or 3rd day postlavage. Lavage also induces variable increases in inflammatory cells depending on the strain used. However, in all three strains examined here (A/J, BALB/c, and C3H/HeJ), inflammatory cell numbers returned to baseline values within 3 days after an initial lavage procedure. The ability to perform repeated BAL in individual mice should prove to be an extremely useful tool in a variety of functional genomic studies in the lung.

longitudinal studies; respiratory compliance; respiratory resistance

THE ABILITY TO SUCCESSFULLY intubate and ventilate individual mice has recently been described by Brown et al. (1). This procedure allows one to make repeated measurements of pulmonary function and airway responsiveness over an extended experimental time course. Such measurements of airway responsiveness in human subjects and animal models are commonly accompanied by bronchoalveolar lavage (BAL) to assess cellular and chemical profiles. Although the BAL procedure can be repeated in most experimental and clinical conditions in both humans (9, 11) and larger animals (2, 7, 12, 15, 17), in mice the procedure is generally considered to be terminal.

In this communication, we sought to develop procedures that would enable repeated BAL in individual mice on multiple occasions. There are several advantages of being able to investigate the cellular profile of individual mice in longitudinal studies, but there have been no reports of BAL having been used in this manner. To achieve this objective, we first developed the procedures that would allow individual mice to survive a whole lung lavage, and then tested whether, on subsequent days, there was an effect of this initial BAL on the subsequent cell profile, lung permeability, and baseline respiratory function. Our results demonstrate that, not only can the repeated lavage procedure be readily carried out in individual mice of different strains, but also that the cell profile and lung mechanics are slightly altered by the lavage itself, taking 2–3 days to recover.

MATERIALS AND METHODS

Mice. We studied 6- to 8-wk-old male A/J mice (Charles River Laboratories, FCRF NCI, Frederick, MD) or C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) and 5- to 8-wk-old BALB/c mice (Charles River Laboratories, Wilmington, MA). Initial mean body weights (±SE) at the start of this study in the A/J, C3H/HeJ, and BALB/c mice were 21.8 ± 0.53, 21.9 ± 0.48, and 20.6 ± 0.52 g, respectively, and these were not significantly different. Animals were housed in laminar flow hoods in an environmentally controlled animal facility for the duration of the experiment and provided with rodent chow and tap water ad libitum.

Intubation and ventilation. Mice were anesthetized with 20–30 mg/kg etomidate and 0.25–0.5 mg/kg fentanyl, then intubated as described by Brown et al. (1). Briefly, mice were suspended by their upper incisors from a rubber band on a 60° incline board. The trachea was transilluminated below the vocal cords to allow visualization of the trachea through the oral cavity. With the lower jaw held open and the tongue held out, a 2-cm length of PE-90 polyethylene tubing with a beveled tip (connected to a 20-gauge needle hub) was gently inserted into the tracheal opening. The mice were ventilated with 100% O_2 at 120 breaths/min with a tidal volume of 200 µl for 3 min before BAL was performed. The ventilatory protocol is shown in Fig. 1. Deep inspirations (DI) were done by inflating the lung to 25 cmH_2O for 4 s beginning after 1 min of ventilation. Baseline resistance and compliance measurements were made 1 min after each DI using the inspiratory occlusion method described by Ewart et al. (4).

Bronchoalveolar lavage and recovery procedure. BAL was performed by slowly delivering 0.6 ml of warm (~37°C) Hanks' balanced salt solution (HBSS) without calcium and magnesium (Biofluids, Rockville, MD), through the tracheal tube. The fluid was slowly withdrawn by gentle suction

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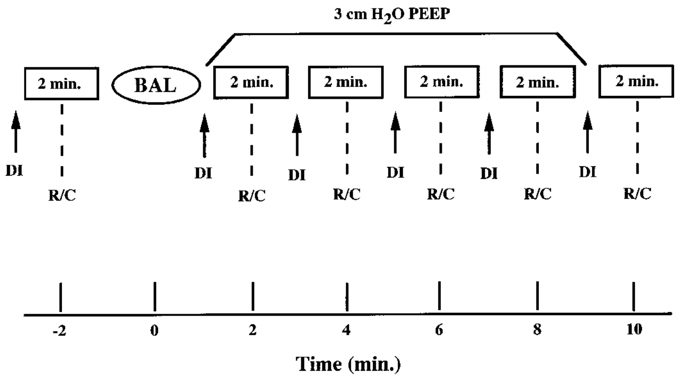

Fig. 1. Experimental protocol showing timing of ventilatory maneuvers [deep inspirations (DI, 25 cmH_2O) and resistance/compliance measurements (R/C)] and bronchoalveolar lavage (BAL). PEEP, positive end expiratory pressure (3 cmH_2O).

immediately after delivery and stored on ice until further processing.

Mice were returned to the ventilator and a positive end expiratory pressure (PEEP) of 2.5–3 cmH_2O was applied. A DI was applied every 2 min for 10–12 min after BAL, followed by resistance and compliance measurements. PEEP was removed ~8–10 min after BAL, and a final DI and respiratory mechanics measurement was made with the animal off PEEP. Mice were extubated and watched carefully until they resumed spontaneous respiration, generally within 1 min following extubation. Animals were allowed to recover from anesthesia on a warming pad, then were returned to their cages. These intubation, ventilation, and BAL procedures were repeated 1, 2, 3, 4, 7, 14, and 21 days after the initial procedure. The same individual mice were used in repeat studies at the 7-, 14-, and 21-day periods. However, because of possible effects of repeating the lavage multiple times with short time intervals, measurements at *days 1–4* after the initial BAL were all in different animals.

Assessment of airway inflammation and injury. Recovered lavage fluid was centrifuged (300 *g* for 8 min), and the supernatant was removed and stored at −80°C for later measurement of total protein content. The cell pellet was resuspended in 0.5 ml 10% FBS. Total cells were counted with a hemocytometer. Slides were prepared by cytocentrifugation (Cytospin 3; Shandon Instruments, Pittsburgh, PA) and stained with Diff Quik (Dade Behring, Düdingen, Switzerland). BAL cell differential counts were determined using morphological criteria under a light microscope with evaluation of ≥500 cells/slide.

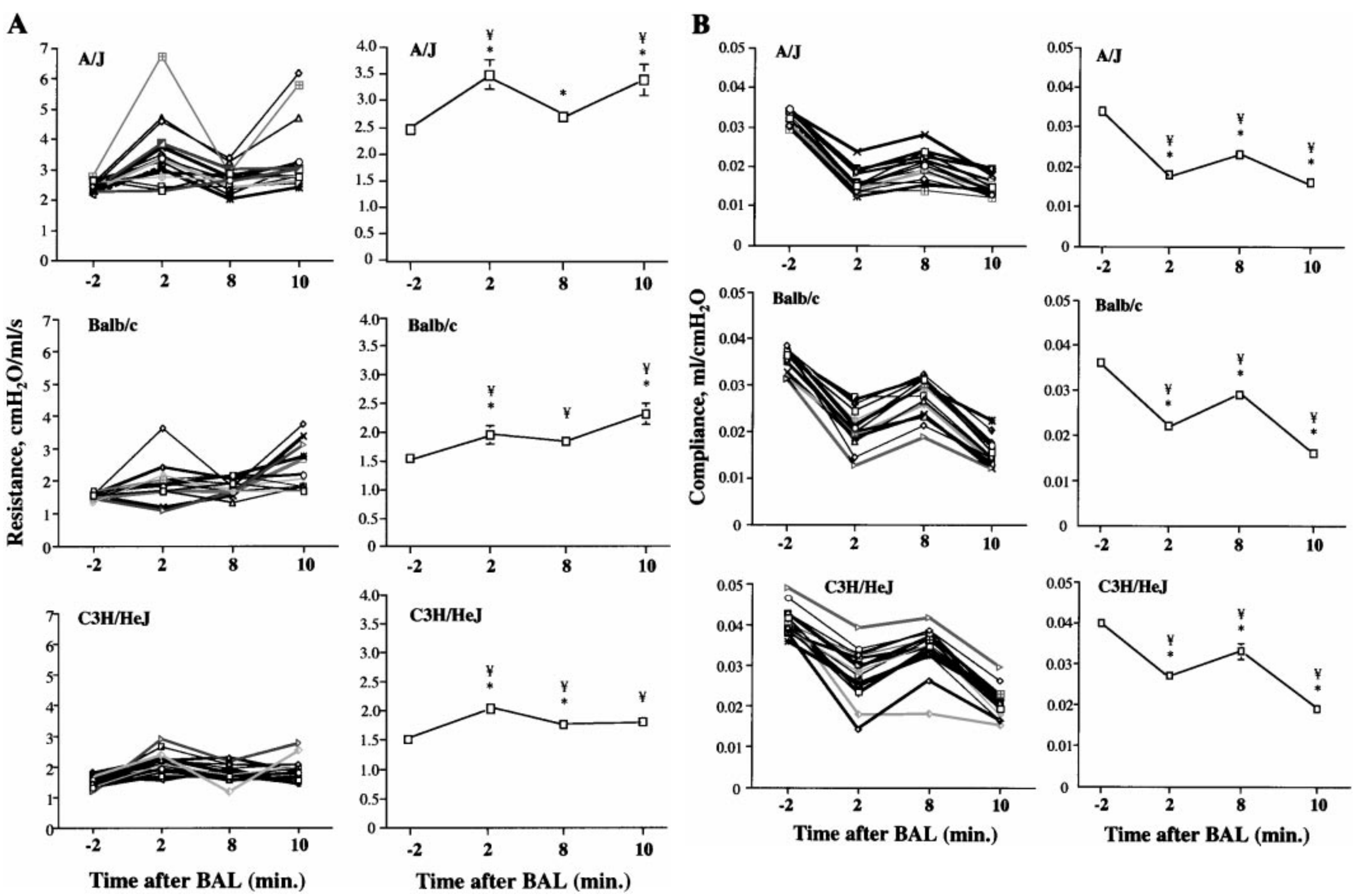

Fig. 2. Resistance (*A*) and compliance (*B*) measurements in A/J, BALB/c, and C3H/HeJ mice before ($t = -2$ min) and after BAL. Values for individual mice are shown on *left*, and mean ± SE for each time point are on *right*. Note that error bars are not visible at most time points on the compliance graph because SE was ≤ 0.001. A/J mice had the highest baseline resistance, as well as the greatest change and range of resistance values 2 min after BAL compared with BALB/c and C3H/HeJ mice. Correspondingly, the A/J strain also had the lowest baseline compliance values of the 3 strains. However, the range of compliance measurements was greater in BALB/c and C3H/HeJ strains. *Significant change from the previous time point. ¥Significant change from prelavage values ($t = -2$ min). See text for further discussion.

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BCA total protein assay (Pierce, Rockford, IL) was performed on BAL supernatants according to manufacturer's instructions. Optical density readings of samples were converted to micrograms per milliliter using values obtained from standard curves generated with serial dilutions of BSA. The limit of detection was 25 μg/ml.

Data analysis. Lung resistance and compliance are reported as individual values for each animal as well as the mean ± SE for each time point. Paired t-tests were used to determine differences between time points with significance assumed at $P < 0.05$. Differential cell counts are reported as individual values for each animal. Total protein values are expressed as the mean ± SE for each time point. One-way ANOVA was used to determine differences between time points with post hoc comparisons by the method of Fisher. Significance was assumed at $P < 0.05$.

RESULTS

Most mice survived this repeated intubation and lavage procedure quite well and were able to complete the entire protocol. We did not attempt to restudy mice any sooner than 1 day following a lavage, but our results demonstrate that mice can be intubated and lavaged again as early as the following day. Not all mice survived the entire study period, and data from the last lavage in these animals were not included. Those that did not survive generally died as a result of accidental injury to the larynx or trachea during one of the intubations. We found that, because the A/J and BALB/c strains were more easily intubated, they had a better survival rate than the C3H/HeJ strain (15/16, 13/15, and 11/18, respectively). Residual lavage fluid in the lungs (see below) did not seem to be the cause of any recovery problems.

Resistance and compliance measurements were made before and after lung lavage. Figure 2*A* shows the respiratory system resistance (Rrs) in individual mice of each strain on the *left* and group means ± SE for each strain on the *right*. Figure 2*B* shows the corresponding individual and mean values for respiratory system compliance (Crs). Rrs and Crs values at the 4 and 6 min time points postlavage were not significantly different from those at 8 min (data not shown). Mean Rrs in all strains increased significantly immediately after lavage but returned toward baseline after the 8-min period with PEEP. With removal of PEEP, Rrs then increased. Of the three strains studied, A/J mice had significantly higher baseline resistance (2.17–2.77 $\mathrm{cmH_2O \cdot ml^{-1} \cdot s^{-1}}$) than the other two strains (Fig. 3, *day 0*) and exhibited the greatest change and range of resistances after lavage. C3H/HeJ and BALB/c mice had baseline resistances of 1.325–1.807 $\mathrm{cmH_2O \cdot ml^{-1} \cdot s^{-1}}$ and exhibited comparatively little change in resistance after lavage. Correspondingly, the C3H/HeJ and BALB/c strains had higher baseline compliance compared with the A/J strain, but all three strains were significantly different (Fig. 3). As with resistance, the changes in compliance while on PEEP were not sustained after its removal.

Figure 3 shows the initial baseline resistances and compliances before lavage at the start of each experimental time point. All strains showed a residual effect

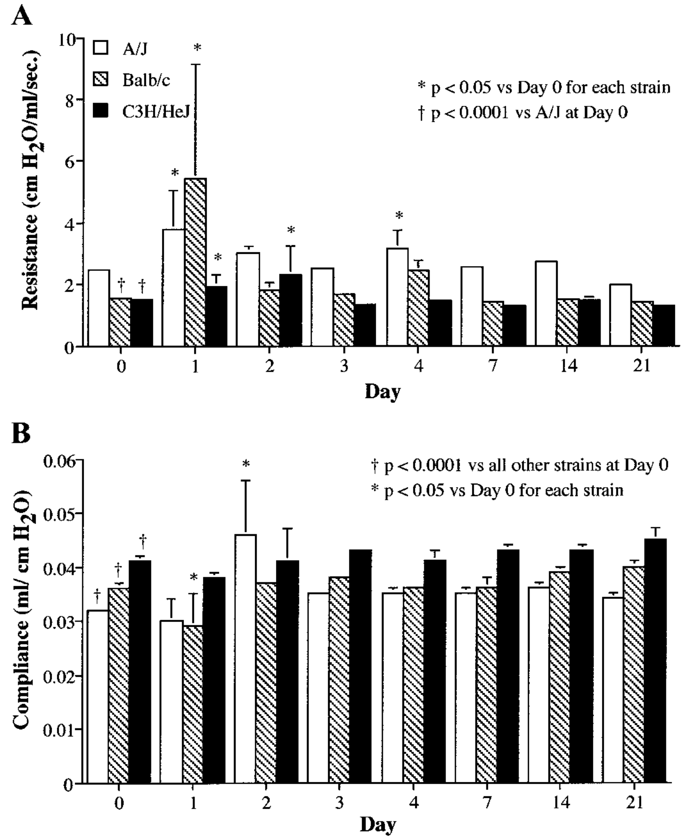

Fig. 3. Resistance (*A*) and compliance (*B*) measurements in A/J, BALB/c, and C3H/HeJ mice at the start of each procedure prior to BAL. Resistance is elevated in all 3 strains 1 day postlavage.

of the previous day's lavage with an increase in resistance. Individual strains showed variable changes in resistance on subsequent days and in compliance on *days 1* and *2*.

Of the 0.6 ml of fluid introduced into the lung during the lavage procedure, ~70% was recovered. The total number of cells recovered from lavage fluid increased mildly 1 day after the initial procedure in two strains but returned to baseline values within 3 days (Fig. 4). These increases in cells recovered on *days 1* and *2* post-BAL can be attributed primarily to increases in neutrophils and eosinophils (Figs. 5 and 6).

In the three strains of mice examined, neutrophil numbers in lavage fluid were increased on *days 1* and *2* after the initial lavage and returned to baseline values within 3–4 days (Fig. 5). All three strains initially had very low numbers of neutrophils; however, the BALB/c and C3H/HeJ strains exhibited ~10-fold greater numbers of neutrophils at *days 1* and *2* than did the A/J strain.

Interestingly, eosinophil numbers also increased markedly 1 day after the initial procedure and declined temporally, returning to baseline numbers by *day 3* (Fig. 6). While all three strains exhibited the same behavior, BALB/c mice appeared to have the greatest increase in eosinophils, followed by C3H/HeJ and A/J mice, respectively. Note that the number of animals at each time point was clearly not sufficient to determine

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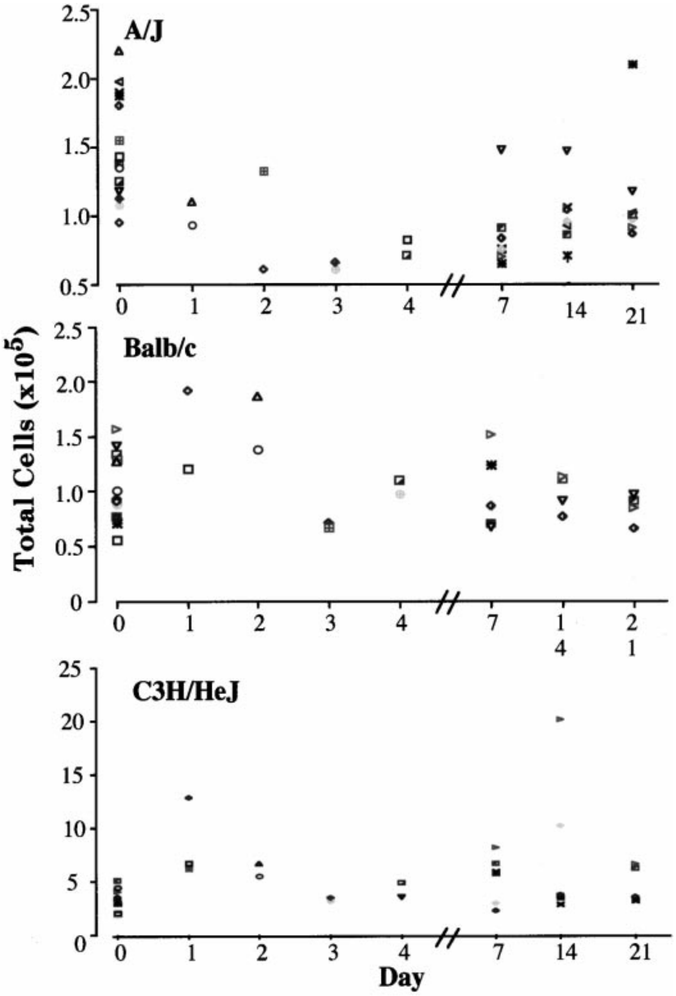

Fig. 4. Total cell numbers recovered in BAL fluid on each of the study days from A/J, BALB/c, and C3H/HeJ mice. Each symbol represents cells from individual mice.

statistical differences between strains in this acute response to lavage. This was by experimental design, as a primary goal was to determine when the lavage cell profile would return to baseline following a single lavage. For this reason the group of mice were studied at distributed time points, rather than larger numbers at just a few fixed time points. The increases in eosinophils seen in a few individual animals at 7, 14, and 21 days after the initial procedure are thought to indicate variability in the development of allergy to antigens in the housing environment. However, it is worth noting that in general, the magnitude of the increase in eosinophils seen following a single lavage is several orders of magnitude less than that seen following an experimental allergic challenge (15).

In contrast to eosinophils and neutrophils, macrophage numbers did not show any significant change from the range of baseline values in the first 4 days after the initial lavage (Fig. 7). The A/J and BALB/c strains exhibited a similar range in the number of macrophages at baseline. In comparison, the C3H/HeJ strain exhibited a wider baseline range of macrophage numbers. In addition, a few individuals of the C3H/HeJ strain exhibited an elevated number of macrophages at 7 and 14 days. Such increases were not seen in either A/J or BALB/c mice.

Likewise, the A/J and BALB/c strains exhibited similar ranges of baseline epithelial cell numbers, and subsequent lavages did not reveal any increase in epithelial cells in these two strains (Fig. 8). On the other hand, the C3H/HeJ strain had a larger range of baseline epithelial cell numbers than observed in the other two strains. Additionally, one C3H/HeJ animal exhibited increased numbers of epithelial cells 1 day after the initial lavage, and several animals had elevated numbers of epithelial cells at 7, 14, and 21 days. Such increases in epithelial cell numbers may possibly reflect mild trauma incurred during intubation in the C3H/HeJ strain.

Total protein in BAL fluid, a measure of lung permeability, increased significantly over baseline values 1 day after the initial lavage in all three strains of mice, although to different degrees in each strain (Fig. 9). Despite similar baseline values in all three strains of

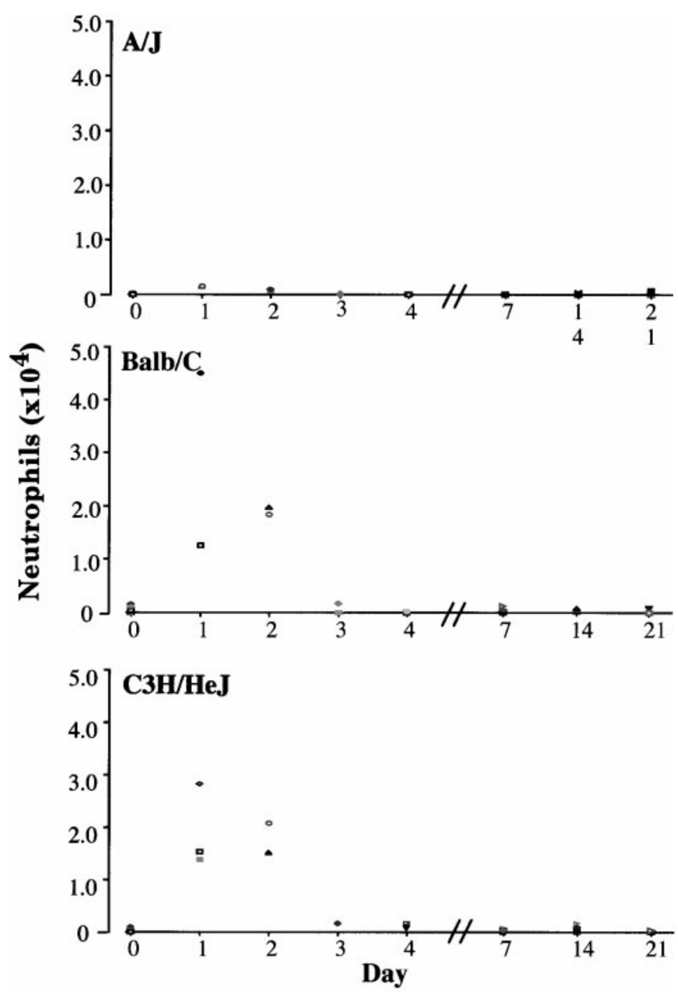

Fig. 5. Neutrophil numbers in BAL fluid from A/J, BALB/c, and C3H/HeJ mice were increased 1 and 2 days after the initial lavage and returned to baseline values 3–4 days post-BAL. Although this trend was observed in all 3 strains, BALB/c and C3H/HeJ mice developed a much greater degree of neutrophilic inflammation than did A/J mice. Each symbol represents cells from individual mice.

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mice, the C3H/HeJ strain exhibited the greatest increase in total protein 1 day after the initial lavage. Interestingly, total protein increased even further in this strain on *day 2*, whereas values decreased in the other two strains at this time point. By *day 3*, total protein levels returned to and remained at baseline values in C3H/HeJ mice. Although total protein levels were increased at various time points in A/J and BALB/c mice, there was no apparent pattern to the increases. Furthermore, although the increases in total protein seen in A/J and BALB/c mice were significantly greater than baseline values, they were also significantly lower than the level seen in C3H/HeJ mice at 2 days postlavage.

DISCUSSION

In the present study, we have developed a procedure by which individual mice can survive whole lung lavage on multiple occasions. Using the intubation technique recently described by Brown et al. (1), three different strains of mice were lavaged repeatedly over a 3-wk period. On each occasion, respiratory system resistance and compliance were measured before and after lavage,

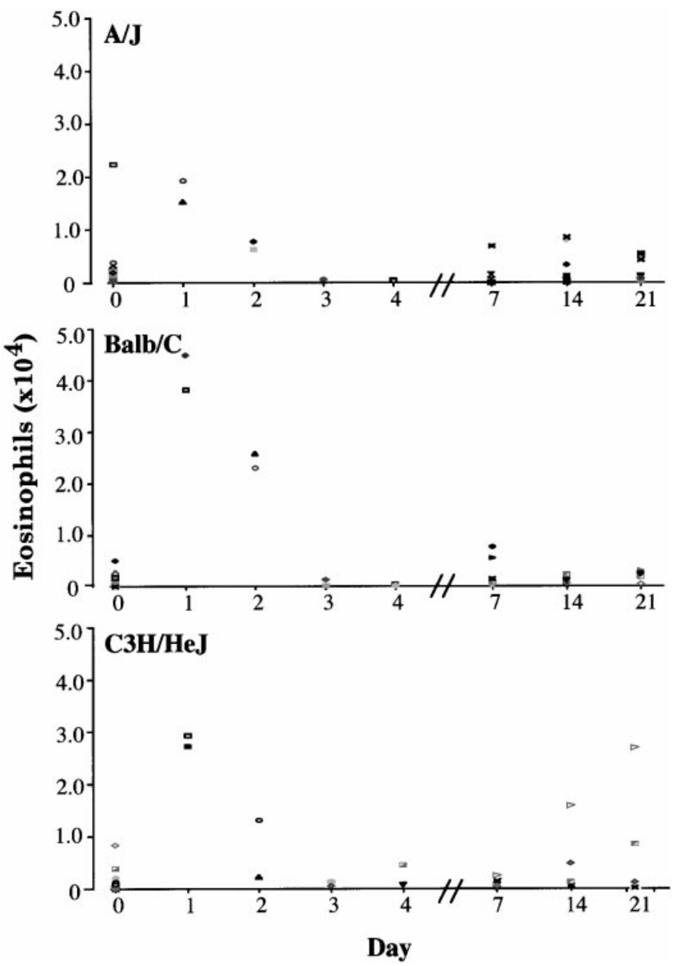

Fig. 6. Number of eosinophils in BAL fluid from A/J, BALB/c, and C3H/HeJ mice increased substantially 1 day after the first lavage, but returned to baseline numbers in a time-dependent manner over the next 2 days. Each symbol represents cells from individual mice.

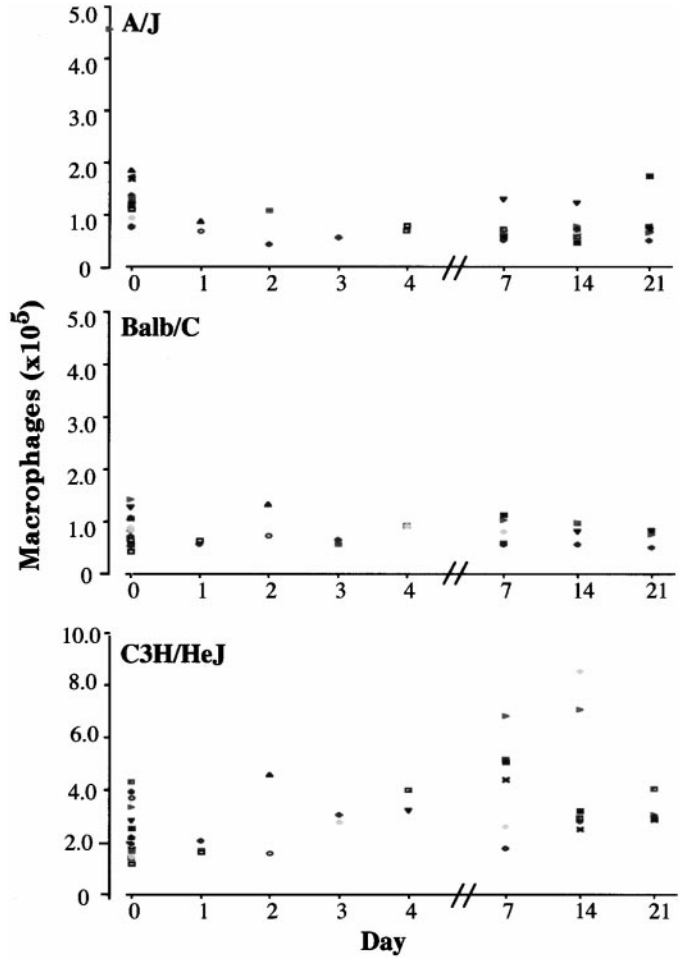

Fig. 7. Number of macrophages recovered in BAL fluid was not affected at any time point following the initial lavage in A/J, BALB/c, or C3H/HeJ strains. C3H/HeJ strain had substantially higher numbers of macrophages at all times. (Note the scale is doubled for the C3H/HeJ strain.) Each symbol represents cells from individual mice.

and changes in the cellular profile, as well as total protein in lavage fluid were assessed.

We found that in all strains of mice studied, respiratory resistance increases immediately after lavage. This increase in resistance may result from smooth muscle constriction or from physical obstruction of the airways by remaining lavage fluid. This residual fluid was substantial, averaging 180 µl, or more than half a mouse's normal functional residual capacity (FRC) (10). Resistance returns toward baseline values after several minutes of ventilation with 3 cmH_2O of PEEP and repeated DI. The DI serve to open airways and alveoli, allowing the residual fluid to move from the airspace lumen to the interstitial space and circulation. Lavage may also dilute lung surfactant, which could contribute to the acute decreases in compliance. This seems less likely, however, since a single saline lavage, as we have done, generally does not cause such major loss of lung stability (5). Upon removal of the PEEP, the compliance again falls, but this does not necessarily imply instability related to loss of surfactant. Recent measurements of chest wall compliance have demonstrated that there is negligible recoil in the physiologi-

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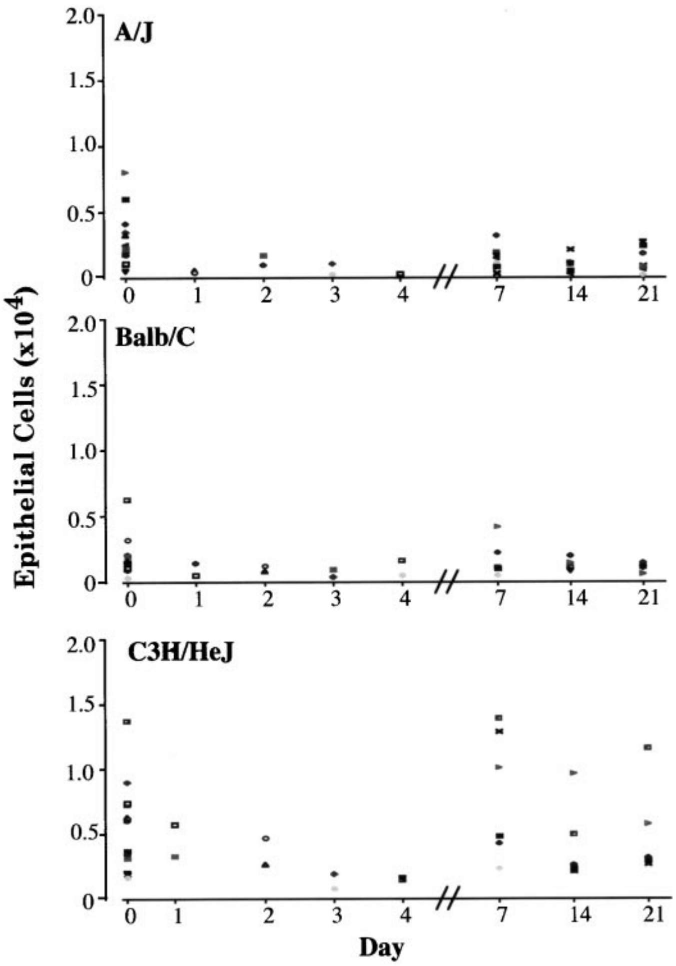

Fig. 8. Number of epithelial cells recovered in BAL fluid was not different from baseline values at any time point following the initial lavage in A/J or BALB/c mice. The increased number of epithelial cells at later time points in some individual C3H/HeJ mice may be related to injury caused by the intubation procedure (see text). Each symbol represents cells from individual mice.

cal range of thoracic volumes (13). It thus seems likely that mice regulate their FRC with some active inspiratory tone, as originally suggested by Leith (8). If so, then even with an intact chest wall, the addition of some PEEP to a ventilated mouse should increase the dynamic lung compliance. We have observed this anecdotally in a few normal mice, where the dynamic compliance increased slightly as the level of end expiratory pressure was increased up to about 3–4 cmH_2O (unpublished observations).

Our present results confirm the higher baseline resistance and lower baseline compliance observed in the A/J strain previously found by Ewart et al. (4). In the study by Ewart et al., only A/J and C3H/HeJ mice were compared, but the present work demonstrates that the BALB/c strain is genetically more comparable to the C3H/HeJ strain at least with regard to respiratory resistance. It is tempting to speculate that the higher baseline resistance and responsiveness in A/J mice may cause the greater increase in resistance seen after lavage. However, Ewart et al. (4) were unable to demonstrate any relation between baseline resistance and sensitivity to methacholine, so validity of this speculation is uncertain.

Genetic differences may also explain the different degrees of inflammation observed in the three strains. The A/J strain is genetically predisposed to the development of Th2 immune responses and is commonly used as a model of allergic asthma (16). This tendency toward Th2-type responses may be responsible for the relatively lower increase in neutrophils in this strain. In comparison, the C3H/HeJ and BALB/c strains tend toward Th1-type responses, which correspond to the greater increases in neutrophils seen in these strains.

The increase in neutrophil number observed in our study is consistent with reports of the effects of lavage in other species. For example, both Weiss et al. (15) and Woodside et al. (17) found that saline lavage results in significant increases in BAL neutrophils that resolve by 3–4 days after the initial lavage in sheep. Similar increases have also been reported in dogs, horses, and monkeys (2, 12, 7). Additionally, Kazmierowski et al. (6) reported the presence of neutrophilic chemotactic factors after repeated lung lavage in monkeys, which may explain the neutrophilic inflammation seen in many species after lavage.

Interestingly, eosinophil numbers also increased in response to lavage in all three strains of mice. Although most studies in other species do not report BAL-induced eosinophils, it is unclear whether eosinophils are not found in other species or are simply not

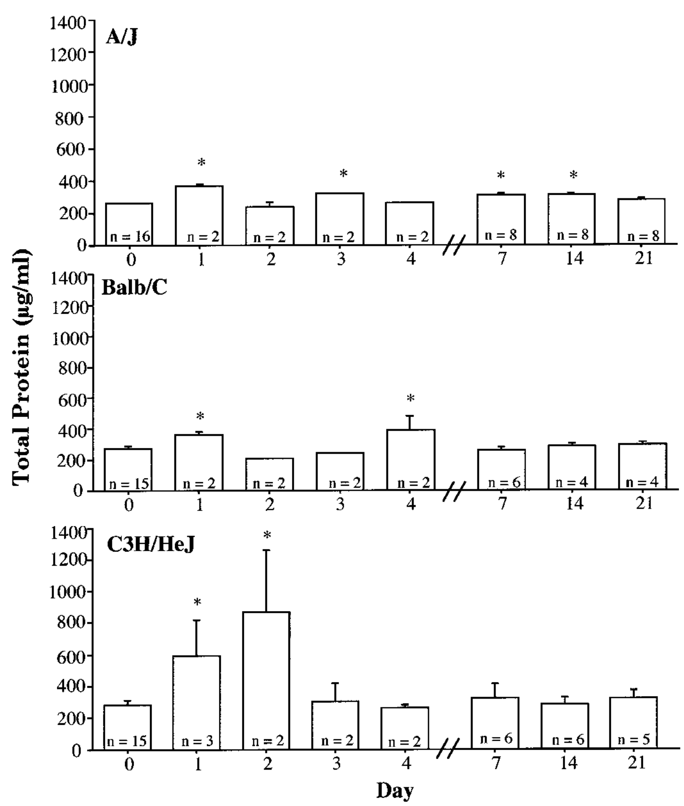

Fig. 9. Total protein levels recovered in BAL fluid at time points following the initial lavage do not exhibit consistent changes in A/J, BALB/c, or C3H/HeJ mice. The greatest increase in total protein is seen in the C3H/HeJ strain 2 days after the initial lavage. Values are means ± SE. *Significant increase over the value at *day 0* (baseline).

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reported. The only report of eosinophil number in BAL fluid after repeated lavage found no change 48 h after the initial lavage in horses (12). Traditionally, eosinophils were thought to be associated only with allergic diseases and parasitic infections; however, recently researchers have noted an eosinophilic response to more general insults such as respiratory exposure to fly ash and ozone (3, 18). Thus eosinophils may play independent protective roles in the lung, separate from those associated with allergic responses. The increase in eosinophils observed during the first few days of the experiment is not thought to be in response to allergen, because the animals used in this study were not in residence long enough to develop allergies. Additionally, most of the eosinophils washed out from this early rise had retained their granules, indicating that they were not activated. The later increases in eosinophils observed in a few individual animals may be attributed to an allergic response, as sensitization to allergen requires ~10–14 days.

In contrast to the increases in neutrophils and eosinophils observed after lavage, macrophage and epithelial cell numbers did not increase over baseline numbers in response to lavage. The literature contains contradictory reports on changes in macrophage numbers after BAL. Krombach et al. (7) reported decreases in macrophage numbers in monkeys, whereas the study by Woodside et al. (17) in sheep showed an increase over 4 days. Because sterile HBSS was used in our study, it is unlikely that any stimulus such as endotoxin was present to activate or recruit macrophages.

Like macrophage numbers, epithelial cell numbers did not increase in response to lavage during the first 4 days after the initial lavage, and in some cases the numbers decreased. It is likely that loose or sloughed epithelial cells in the airways were washed out in the first lavage, resulting in decreased numbers in subsequent lavages. As noted earlier, the intubation process itself likely caused the increase in epithelial cells 1 day after the initial lavage in one C3H/HeJ mouse.

Total lavagable protein was measured to assess permeability changes that may result from the procedure. All three strains exhibited a significant increase in total protein 1 day after the initial lavage. However, the pattern of changes in total protein levels thereafter was inconsistent between strains. C3H/HeJ mice exhibited an even greater level of protein on *day 2*, whereas protein levels in A/J and BALB/c mice returned to baseline values. However, the number of mice used at each early time point precludes determination of significant differences among strains.

Although repeated BAL procedures have been commonly used in larger species including humans, in small species such as mice, use of BAL has been limited to a single postmortem time point per individual. Because the procedure we describe is an in vivo procedure, from which the mice recover, it may be more relevant to human BAL than postmortem lavages. Recently, Varner et al. (14) described a technique of serial segmental lavage in rats, but lavages subsequent to the initial lavage were conducted at 2-wk intervals, at which time there were no changes in cellular profile or pulmonary resistance from baseline. Several factors, however, make the mouse a valuable model for the study of various diseases, including the availability of numerous well-characterized inbred strains, the ability to express or delete specific genes, and the wealth of specific proteins available for experimental studies in mice.

In summary, we have described a procedure that allows individual mice to survive whole lung lavage on multiple occasions. The lavage procedure itself results in immediate increases in respiratory system resistance and concomitant decreases in compliance, but these parameters return to prelavage values by the 2nd or 3rd day postlavage. Lavage also induces variable increases in inflammatory cells depending on the strain used. However, in all three strains examined here (A/J, BALB/c, C3H/HeJ), inflammatory cell numbers returned to baseline values within 3 days after an initial lavage procedure. This ability to repeatedly conduct BAL in individual mice should prove to be an extremely useful tool in a variety of functional genomic studies in the lung.

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