Serial segmental bronchoalveolar lavage in individual rats

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We have developed a Brown Norway rat model of virus-induced airway dysfunction that resembles human asthma in several ways: intermittent airflow obstruction that resolves spontaneously with time or corticosteroids, airway hyperresponsiveness to methacholine, inflammation characterized by eosinophils and mast cells, and airway remodeling involving subepithelial fibrosis and airway wall thickening (2). We previously developed methods for repeated measures of pulmonary physiology (2), but correlates between physiology and inflammation were possible only for single time points because whole lung bronchoalveolar lavage (BAL) is a terminal procedure. Therefore, we wanted to develop a technique that would allow us to sample the airway intermittently for inflammatory cells without affecting the natural course of each animal’s condition. The goal was to develop a method of segmental lung lavage that would provide information analogous to that of whole lung lavage but that could be performed repeatedly over time without causing harm to the animal’s lungs.

METHODS

Animals. All methods and procedures were approved by the Animal Care and Use Committee of the University of Wisconsin. Data were obtained from 79 adult male Brown Norway rats (Charles River Laboratories, Kingston, NY): 49 from a longitudinal study of airway function during the postviral asthmalike syndrome (3) and 30 from protocols involving pulmonary antigen challenge 2–4 days before the study. The rats weighed 291 ± 30 (SD) g on the day of lavage studies.

Pulmonary physiology measurements. An oral tracheal tube (PE-240, ID 1.7 mm, OD 2.4 mm, length 5 cm; Becton-Dickinson, Sparks, MD) was placed, under direct visualization, after light pentobarbital sodium anesthesia (35–45 mg/kg ip), as described previously (2). After placement of the tracheal catheter, the rats were positioned in a constant-pressure rodent plethysmograph and were allowed to breathe spontaneously to the outside. Changes in transpulmonary pressure were measured with a water-filled PE-160 esophageal cannula attached to a pressure transducer (model MPX-11DP, Motorola). The esophageal cannula was positioned in the lower esophagus at a point having a distinct cardiac artifact and negligible differences between esophageal and tracheal pressure changes during an occluded inspiratory effort. Pulmonary resistance was measured as described previously (2), by using a pulmonary mechanics analyzer (model 6, Buxco Electronics, Sharon, CT).

Segmental BAL. A PE-50 catheter (ID 0.58 mm, OD 0.97 mm, length 12 cm; Becton-Dickinson) was passed through the tracheal tube, advanced to a wedge position, and held in place by the force of a slight bend in the external portion of the catheter. A 0.1-ml volume of warm sterile buffer solution (Hanks’ balanced salt solution without calcium or magnesium; Life Technologies, Grand Island, NY) was instilled, followed by 0.2 ml air, and then withdrawn with gentle suction, and this procedure was repeated for a total of five lavages. Withdrawal of the catheter slightly during suction facilitated the return of lavage fluid to the syringe. The presence of foamy air bubbles in the lavage fluid was a reliable indicator of an adequate sample.

Leukocyte differential counts were obtained from 200 cells on a Cytospin-3 (Shandon Lipshaw, Pittsburgh, PA) slide stained with Diff-Quik (Baxter Healthcare, Miami, FL) under oil immersion by using standard morphological criteria; all cell counts were done by one investigator under blinded conditions. For analyses, macrophages, monocytes, and lymphocytes were pooled into a single mononuclear cell category.
Quantitative total nucleated cell counts were measured in unprocessed lavage fluid from 16 rats, by using a Hemo-W cell counter (Coulter Electronics, Hialeah, FL); in preliminary trials the results from this method were comparable with those obtained by the hemacytometer method. Total individual leukocyte counts were computed for each lavage sample by multiplication of differential percents by nucleated cells per milliliter by sample volume.

Whole lung BAL. After completion of physiological studies and segmental BAL, rats were given additional anesthetic and exsanguinated by cardiac puncture. Whole lung quantitative BAL was performed by multiple inflations to total lung capacity with cold buffer, by first connecting the tracheal cannula to a buffer reservoir elevated 15–20 cm above the level of the lungs and then draining by opening the stopcock to a collection container placed slightly below lung level for a total of five exchanges. The lavage fluid was centrifuged, and the cell pellet was resuspended in 1.0 ml of buffer. Total and differential leukocyte counts were obtained as for the segmental BAL.

Data analysis. Statistical analysis was done by using Systat version 7.0 software (SPSS, Chicago, IL). The percentages of mononuclear cells, neutrophils, and eosinophils were compared for the segmental and whole lung lavage samples by using the approach of Bland and Altman (1) to assess absolute differences between the methods relative to the mean value of the two measurements. It was not possible to determine confidence intervals for the differences by parametric techniques, because of nonnormal distributions for the variables. Instead, the differences were described as medians, along with the interquartile (25–75th percentile) ranges. Because of apparent changes in patterns during acute inflammation compared with normal control conditions, subgroups were identified according to whether acute inflammation was present; this was defined as the presence of >10% granulocytes (neutrophils + eosinophils) in the total lung BAL sample. The Spearman rank correlation coefficient (\( r_s \)) was used to test the degree of correlation between variables that did not meet parametric assumptions. The Wilcoxon signed-ranks test was used to test for differences between the percentages of cells obtained by the two lavage methods in each rat. Total neutrophil counts in segmental and whole lung samples conformed to the assumptions for linear regression analyses after a log transform was applied; the variability of the segmental sample measurement around the predicted value was expressed as the coefficient of determination (\( R^2 \)) and the SE of the estimate (SEE). Repeated-measures ANOVA was used to test for systematic changes in BAL neutrophils and pulmonary resistance in longitudinal studies.

**RESULTS**

The volume of BAL fluid obtained was 48 ± 5.5 (SD) ml for the whole lung method and 0.26 ± 0.07 ml for the segmental method. For 16 rats in which the total number of nucleated BAL cells was determined for both BAL methods, the median cell counts were 43.5 × 10^5 for whole lung BAL and 2.3 × 10^5 for segmental BAL. Most samples contained small numbers of erythrocytes, with no apparent difference in the two lavage techniques with respect to erythrocyte density on the cytocentrifuge slides. The segmental samples had relatively greater numbers of epithelial cells on the cytocentrifuge slides compared with the whole lung samples.

Location of segmental BAL. In preliminary studies, insufflation of 0.1 ml india ink through the lavage catheter in a wedge position revealed a small wedge-shaped area of staining in the base of the left lung. In the majority of study rats, gross examination of the lungs immediately after the segmental BAL was performed allowed determination of the wedge position of the catheter from the color change due to residual lavage fluid in that area of the lung. The left lung base was the site of segmental lavage in 78% of those examined, the remainder being in the right middle lobe (14%) or not found by gross examination of the lungs (8%). In later trials, directing the curvature of the lavage catheter to the left during insertion resulted in nearly uniform left lung base location of the lavage.

Comparison of segmental and whole lung BAL. Figure 1 compares the percentages of mononuclear cells (mostly macrophages and monocytes, with few lymphocytes), neutrophils, and eosinophils in whole lung vs. segmental BAL in 79 rats. Overall, the cell proportions correlated for the two BAL methods (\( r_s = 0.73, 0.67, \) and 0.80 for mononuclear cells, neutrophils, and eosinophils, respectively), although there were deviations from the line of identity that suggested the presence of population differences as well as random variability (Fig. 1). The agreement between the two BAL methods was assessed by using the approach of Bland and Altman (1), as illustrated in Fig. 2. Both the magnitude of differences between the paired measurements and the direction of the differences were influenced by the presence of an acute inflammatory process, defined as >10% granulocytes (neutrophils + eosinophils) in the whole lung BAL. In 52 rats without acute inflammation, there were no significant differences between the proportions of mononuclear cells (\( P = 0.26, \) Wilcoxon signed ranks) or of neutrophils (\( P = 0.18, \) Wilcoxon signed ranks) in segmental vs. whole lung BAL, and the absolute differences were small, the interquartile (25–75th percentile) ranges being 1.1–10.2 for the percentages of mononuclear cells, 5.5–10.2 for neutrophils, and 4.5–8.6 for eosinophils.
75 percentile) ranges being −1.5−2.0% for mononuclear cells and −1.0−2.0% for neutrophils (Fig. 2). The percentage of eosinophils was slightly lower (median 0.5 percentage points; \( P < 0.0001 \), Wilcoxon signed ranks) in the segmental BAL compared with the whole lung BAL from this subgroup, but there was a high level of agreement between the segmental and whole lung results, the interquartile range being −1–0% for the absolute differences. In 29 rats that had >10% granulocytes in the whole lung BAL, the segmental BAL had a larger proportion of mononuclear cells (median difference = 3%; \( P = 0.017 \), Wilcoxon signed ranks) and a smaller proportion of neutrophils (median difference = −6.5%; \( P = 0.006 \)) compared with whole lung BAL (Fig. 2). The variability of the differences between the BAL methods also increased markedly for mononuclear cells and neutrophils from rats with acute inflammation, with interquartile ranges of −0.5–12.5 and −14–1.0, respectively. There also was more variability between the two measures of BAL percentage of eosinophils in the rats with acute inflammation, the interquartile range for absolute differences increasing to −3.5–5, but there was no bias detected (median difference = 0.0; \( P > 0.5 \)). Other than four rats that had marked increases in segmental BAL eosinophilia that were not matched in whole lung BAL, the overall agreement between segmental vs. whole lung BAL percentage of eosinophils measurements remained good in the rats with acute inflammation (Fig. 2).

Total BAL cell counts were obtained for both segmental and whole lung BAL in 16 rats, and these data were used to compare variability in absolute cell counts for the two methods (Fig. 3). There was a >10-fold range in numbers of mononuclear cells obtained by segmental BAL, compared with a <3-fold range in whole lung samples, and there was no correlation between the two measurements (\( r_s = −0.02 \)). In contrast, there was a >100-fold range in neutrophil numbers obtained by both methods, and the two measurements varied linearly over the entire range of log-transformed values (\( R^2 = 0.63 \), SEE = 0.44 log units). Eosinophils exhibited a similar pattern as neutrophils, although a linear model was not applicable to describe the variability of segmental eosinophils with whole lung eosinophils (\( r_s = 0.64 \)).

Repeated studies over time. We studied 10 normal (no infections or allergen challenges) rats on three occasions over a 4-wk period of time to determine the level of random variability in segmental BAL cell counts and to determine whether repeated studies may cause pulmonary inflammation or airway obstruction. Figure 4 summarizes the pulmonary resistance and the percentage of neutrophils in the segmental BAL measured in the 10 individual rats at the 3 time points. Except for two isolated measurements, <5% neutrophils were present in the segmental BAL samples, and there was no significant change in neutrophil count associated with repeated studies (Fig. 4; repeated-measures ANOVA). There also was no significant increase in pulmonary resistance associated with repeated studies at 2-wk intervals (Fig. 4; repeated-measures ANOVA).

DISCUSSION

These studies indicate that it is feasible to use a segmental BAL technique to monitor inflammatory cells in the airways of small animals in a longitudinal manner. However, the results also illustrate that during acute inflammation the measures of cell populations may differ considerably between samples obtained via segmental lavage vs. total lung lavage. One potential explanation for these differences is that pul-

Fig. 2. Absolute differences between percentages of cells in segmental vs. whole lung samples plotted against mean value from the 2 samples. Line of zero difference is shown on each plot. A: mononuclear cells; B: neutrophils; C: eosinophils. ○, Rats having ≤10% \( \Delta \), rats having >10% granulocytes in their whole lung BAL.

Fig. 3. Comparison of total numbers of mononuclear cells (A), neutrophils (B), and eosinophils (C) obtained via segmental vs. whole lung BAL in 16 rats. Line is least squares linear fit for log-transformed data (2 eosinophil points with values of 0 are not shown).
Pulmonary inflammation typically is not homogeneous throughout the lung and that the limited area sampled by the segmental technique may not be representative of the whole lung. Examples of this scenario may be the outliers in the eosinophil plot in Fig. 2, where the percentage of eosinophils in the segmental sample was elevated disproportionally compared with that in the whole lung lavage. If random regional variations in inflammation were the predominant reason for differences between the two measurements, however, we would predict that the median difference would be zero. Although this appears to hold true for the percentage of eosinophils, there appears to be an additional nonrandom factor creating a bias for measures of mononuclear cells and neutrophils. The percentage of mononuclear cells was significantly higher and the percentage of neutrophils was significantly lower in the segmental samples compared with the values of total lung lavages. Because the cell counts are proportions, a bias in one cell type must necessarily create an opposite bias in at least one of the other cell types; thus a real sampling difference either for mononuclear cells or for neutrophils would be sufficient to explain the observation. Eosinophils, the least populous of the three, would be affected to a less noticeable extent. One potential explanation for sampling differences might be the inflammatory cell populations residing in the central airways, which are included in whole lung lavage but are bypassed for segmental lavage. Examination of the number of cells obtained with each technique (Fig. 3) suggests that the bias in cell proportions may have originated with sampling differences with respect to mononuclear cells, in that total neutrophils correlated over a broad range with the two lavage techniques, whereas mononuclear cell numbers did not.

Although segmental lavage appears to sample a population of cells with proportions different from those of whole lung lavage during acute inflammation, it may yet have considerable utility for longitudinal sampling of a defined area of the lung. The anatomy of the rat’s central airways makes the left lung base a high probability for blind cannulation, and having a slight curvature in the catheter directed leftward during insertion makes cannulation of other lung lobes (which in the rat are all served by the right mainstem bronchus) rare. As shown in Fig. 4, stability of background cell counts over repeated studies should allow detection of a sequence of events during acute inflammation.

We have described a technique in rats that permits intermittent monitoring of the airways for inflammation. Although rodents are used extensively for the study of inflammation in models of pulmonary diseases, they have not been useful for longitudinal studies of inflammatory events in individual animals, because of the lack of a method to obtain lavage samples in nonterminal studies. The ability to measure pulmonary physiology and to obtain BAL samples simultaneously and repeatedly in a longitudinal study design would be of great value for the study of the time course of chronic lung and airway disorders. Although the rat is particularly suitable for this segmental BAL technique, in terms of the size and geometry of its upper airways and lungs, and its resilience to the insults of anesthesia and instrumentation, the method probably could be adapted to other laboratory animals as well.

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REFERENCES


![Fig. 4. Percentages of neutrophils in segmental BAL fluid (A) and pulmonary resistance (B) for 10 rats studied at 3 time points. Each rat is represented by same symbol on both plots.](http://jap.physiology.org/Downloaded from 10.220.33.1 on October 30, 2017)