Efficiency of airway macrophage recovery by bronchoalveolar lavage in hamsters: a stereological approach

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Efficiency of airway macrophage recovery by bronchoalveolar lavage in hamsters: a stereological approach. M. Geiser, A.L. Serra, L.M. Cruz-Orive, M. Baumann, V. Im Hof, P. Gehr. ©ERS Journals Ltd 1995.
ABSTRACT: Macrophages play a central role in the defence of the respiratory tract against deposited particles. In addition to the well-studied alveolar macrophages, airway macrophages have been recognized as an important clearance factor. Bronchoalveolar lavage (BAL) has been used for functional and morphological investigations of macrophages *in vitro*, assuming that all macrophages are removed with equal probability from the lung surface. Airway macrophages have been found in close contact with the epithelial cells. These macrophages may not be easily removed by lavage, and they might constitute a functionally different macrophage population. We have tested the hypothesis that there exists a population of macrophages in the conducting airways that resists removal by lavage.

We lavaged the lungs of four hamsters and fixed the lungs, thereafter, by intravascular perfusion. The number of macrophages in the intrapulmonary conducting airways was estimated with an unbiased stereological technique, the fractionator, and compared to the number of macrophages in the airways of four hamsters whose lungs had not been lavaged prior to fixation.

This *in situ* study revealed that, in hamster lungs, 42% of the airway macrophages were not removed by BAL and that about 5% of all macrophages in the BAL fluid were airway macrophages. Additionally, ultrastructural alterations of the airway epithelium were found.

It is concluded that there exists a population of airway macrophages that resists lavage. This is an aspect which has to be considered in studies performed with macrophages obtained by BAL, since they could represent a functionally different macrophage population.
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Macrophages in the lung are essential in the defence of the respiratory tract against toxic particles and infectious agents [1, 2]. In addition to the well-studied alveolar macrophages, airway macrophages are recognized as an important clearance factor. Even though airway macrophages have been reported to account for only 2–3% of all macrophages on the inner lung surface [3], their relative abundance and their functional roles have been suggested to be similar to those of alveolar macrophages [2]. Phagocytosis of particles has been described in the bronchial tree of mice [4], and in the trachea of rats [5]. We have shown by *in situ* studies that macrophages are recruited to the sites of deposition and phagocytose particles in the intrapulmonary conducting airways of hamsters [6]. Lavage of the trachea and the extrapulmonary main stem bronchi have been used for morphological, biochemical and functional studies of airway macrophages *in vitro* [7, 8]. However, it is uncertain how representative lavaged cells are of the total surface macrophage population [9]. Whereas some authors suggest that a substantial proportion of macrophages resist the removal by lavage [4, 10], LEHNERT *et al.* [7] examined tracheae by scanning electron microscopy and found that lavage effectively removed the cells from the surface. Since we and others have found the macrophages of the intrapulmonary conducting airways in close association with the epithelium [4, 6], we hypothesized that those macrophages might resist removal by lavage, and, hence, would not be available for *in vitro* studies. To further elucidate the role of airway macrophages, it is essential to know whether all these macrophages are removed from the lung surface with equal probability or whether there exists an airway macrophage population that resists lavage, and which might be functionally different from those easily removed by lavage. Thus, it was the aim of our study to estimate the influence of bronchoalveolar lavage (BAL) on the number of macrophages in the intrapulmonary conducting airways.

Materials and methods

Experimental design

The lungs of four animals were lavaged, and, thereafter, fixed by intravascular perfusion (BAL group). The number of macrophages in the intrapulmonary conducting airways was estimated by an unbiased stereological method, the fractionator [6, 11], and compared to the number of airway macrophages of four hamsters whose lungs had not been lavaged prior to fixation (Control group). In addition, the morphology of airway macrophages and of airway epithelial cells was studied qualitatively by light and electron microscopy.

Animals

Male Syrian Golden hamsters (FUME LakIbm:FUME, SPF; Biological Research Laboratory, Füllinsdorf, Switzerland), weighing 127–150 g, were used in this study (table 1). The animals were fasted 12 h prior to the experiment. Otherwise, they had access to food and water *ad libitum*. Anaesthesia was achieved with *i.p.* injections of ketamine-hydrochloride followed by barbiturate [6]. The lungs were then prepared for intravascular perfusion fixation as described previously [13]. Briefly, the deeply anaesthetized hamsters were anticoagulated with an *i.p.* injection of 1,000 IU heparin, intubated for artificial ventilation and, following thoracotomy, the pulmonary artery was cannulated. After perforation of the left heart auricle, the lung circulation was

flushed with a plasma substitute. Thereafter, the lungs of the BAL group animals were lavaged prior to lung fixation.

Bronchoalveolar lavage (BAL)

The lungs were lavaged with 10 × 5 mL divalent cation-free chilled phosphate-buffered saline (PBS; Amimed, Muttenz, Switzerland). For this purpose, the tracheal cannula was connected to a glass syringe and ten 5 mL aliquots of fluid were instilled and recovered within a 7–10 min period (table 1). The lungs were gently massaged during each wash. Following BAL with PBS, the lungs were lavaged with 5 mL of 2% bovine lipid extract surfactant (BLES) [14] to facilitate unfolding of alveoli when the lungs were inflated for subsequent fixation. The recovered BAL fluid was centrifuged, and the number of macrophages in the pellet (N(MBAL)), estimated using a Neubauer® haemocytometer chamber (table 1). The macrophages can easily be recognized by size and shape. Cytology of the lavaged cells was additionally confirmed on Giemsa stained smears.

Lung fixation

After three slow inflation-deflation cycles, the lungs were inflated once more to total lung capacity (TLC) with a pressure of 25 cmH$_2$O column, and then adjusted to about 60% TLC by reducing the inflation pressure to 5 cmH$_2$O (on the deflation limb). Thereafter, the lungs were fixed by intravascular perfusion of phosphate-buffered 2.5% glutaraldehyde, cacodylate-buffered 1% osmium tetroxide, and maleate-buffered 0.5% uranyl

Table 1. – Summary of physiological and bronchoalveolar lavage (BAL) data*

Hamster No.	Body mass g	Lung volume mL#	Time from tracheotomy till fixation min	N(MBAL)	BAL fluid recovery mL	Time used for lung lavage min
Control group†						
1	150	6.52	42			
2	142	6.66	41			
3	138	8.12	39			
4	143	7.77	37			
Mean	143	7.27	40			
SD	5	0.80	2			
BAL group						
5	127	3.78	57	980,000	42	10
6	127	4.49	47	250,000	37	9
7	132	2.87	42	1,030,000	38	8
8	135	3.22	53	1,690,000	40	7
Mean	130	3.59	50	990,000	39	9
SD	4	0.71	7	590,000	2	1

*: the lungs of Syrian Golden hamsters were either lavaged before fixation by intravascular perfusion fixation (BAL group), or fixed only (Control group). The number of harvested macrophages in the recovered BAL fluid, N(MBAL), was assessed using a Neubauer® haemocytometer chamber. †: data from [6]. #: estimated by the water displacement method [12].

acetate, applied in sequence [13]. The fixed lungs were carefully removed from the thorax and their volumes estimated by water displacement [12], although this was not required for the application of the fractionator (table 1).

Stereology and statistics

For unbiased stereological estimates of the total number of macrophages in the intrapulmonary conducting airways, the variant of the fractionator described earlier [6, 11] was used. The tissue sampling included three stages. In the first stage, the lungs, separated into left lung, right lung and accessory lobe and embedded in agar, were cut perpendicularly to the longitudinal axis into 3, 1.5, 1.5, 3, 1.5, 1.5, ... mm thick slices. All 3 mm slices were selected for counting macrophages at the light microscopic level; this made the first sampling fraction, $1/f1$. The 1.5 mm slices were used for electron microscopic studies. In the second stage, each 3 mm slice was embedded in paraffin and, thereafter, cut exhaustively parallel to the slice faces into series of 150 thicker sections (nominal thickness $T = 10$ μm), followed by 10 thinner sections (nominal thickness $T' = 3$ μm). From the latter, four consecutive sections were chosen to make a primary disector (nominal thickness 3×3 μm). They were used as reference and look-up sections [15], with two check-up sections in between to avoid overlooking macrophages. This gave the estimate of the second sampling fraction, est$1/f2$. A large magnification was necessary to identify the macrophages. Therefore, in a third step, subsamples (subdisectors) of the total reference area (lumen plus wall of intrapulmonary airways) had to be taken systematically from each primary disector. The total reference area as well as the subsampled reference area were estimated by point counting. Their ratio gave the estimate of the third sampling fraction, est$1/f3$. On these subdisectors, the airway macrophages (specifically their nuclei), were counted using a light microscope (Vanox AH-2, Olympus, Tokyo, Japan)

at a magnification of 1,000×. The total number of airway macrophages, est$N$(M), was calculated by multiplying the total number of counted airway macrophages, Q^-, with the reciprocals of the three sampling fractions:

$$\text{est}N(\text{M}) = f1 \times \text{est}f2 \times \text{est}f3 \times 1/2 \ (Q_1^- + Q_2^-) \quad (1)$$

The factor 1/2 was introduced because two counts were made on each disector.

The airway macrophage numbers of the two animal groups (BAL and Control groups) were compared using a two-sample Kruskal-Wallis test. Error variance analyses were performed according to the method described previously [6, 11, 16]. For a group of animals in which the total number of macrophages, N, in a given compartment is estimated by an unbiased method, the following equation applies:

$$\text{CV}^2(\text{est}N) = \text{CV}^2 (N) + \text{meanCE}^2 (\text{est}N) \quad (2)$$

The estimate of the total variation among animals is given by the square coefficient of variation of the estimator of N among animals ($\text{CV}^2(\text{est}N)$). The total variation among animals is the sum of the biological variation, *i.e.* the square coefficient of variation of the true N among animals ($\text{CV}^2(N)$), and the error variation due to stereology only, *i.e.* the average over animals of the square coefficient of error within animals ($\text{meanCE}^2(\text{est}N)$). For the estimate of $\text{meanCE}^2(\text{est}N)$ the following predictor was used:

$$\text{meanCE}^2(\text{est}N) \ = \ \frac{1}{3n} \ \sum_{i=1}^{n} \left(\frac{Q_{oi} - Q_{ei}}{Q_{oi} + Q_{ei}} \right)^2 \quad (3)$$

To apply the preceding formula, the tissue slices from the first sampling stage had to be split into two subsamples, the odd- and even-numbered slices. The total number of macrophages in the slices with odd numbers, Q_o, and in the slices with even numbers, Q_e, were scored separately, making the identity $Q_o + Q_e = Q^-$. The results are presented in table 2.

Table 2. – Summary of stereological data*

Hamster No.	$f1$	est$f2$	est$f3$	Q_1^-	Q_2^-	est$N$(M)	Q_o	Q_e	CE(est$N$)	CV(est$N$)	CV(N)
Control group[†]											
1	2	162	18	19	11	89,000	21	9	0.23		
2	2	162	18	13	13	75,000	12	14	0.04		
3	2	162	18	14	17	91,000	13	18	0.09		
4	2	162	16	15	6	53,000	10	11	0.03		
Mean						77,000			0.13	0.23	0.19
SD						17,500					
BAL group											
5	2	173	10	13	15	48,000	6	22	0.33		
6	2	173	9	7	11	29,000	14	4	0.32		
7	2	173	9	10	7	26,000	16	1	0.51		
8	2	173	9	9	7	25,000	3	13	0.36		
Mean						32,000			0.39	0.34	0
SD						10,800					

*: three stage sampling with the sampling fractions, $1/f1$, $1/\text{est}f2$, $1/\text{est}f3$. Macrophage counts, Q_1^-, Q_2^-, were made on subdisectors. The total number of macrophages (est$N$(M)) in the slices with odd numbers and even numbers was tested by estimated coefficient of error (CE) and of variation (CV), using the counts Q_o and Q_e. [†]: data from [6].
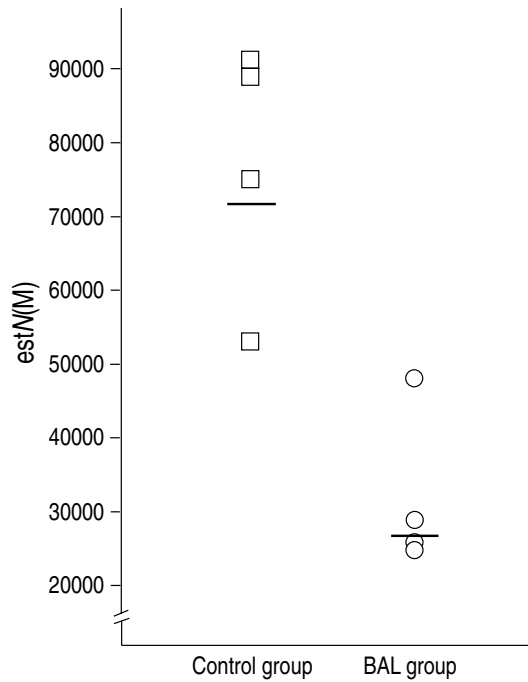
Fig. 1. – Estimated total number of airway macrophages (est$N$(M)) in the intrapulmonary conducting airways of lavaged hamsters (BAL group), and in untreated animals (Control group). Each data point (circle or square) represents one animal, the bars represent the group means. BAL: bronchoalveolar lavage.

Electron microscopy

For qualitative structural analysis, additional tissue blocks were embedded in Epon and ultrathin sections were cut. These sections were examined with a Philips 300 electron microscope at 60 kV.

Results

Intravascular perfusion fixation of the lungs after BAL

All lungs were inflatable and completely perfusable after lavage. However, the lung volume was significantly ($p<0.05$, Wilcoxon signed rank test) smaller in the BAL group animals, even when the volume was corrected for weight (table 1).

Influence of BAL on number of macrophages in airways

The number of macrophages in the intrapulmonary conducting airways (est$N$(M)), was significantly, 2.4 fold, smaller ($p<0.05$) in the BAL group than in the Control group (table 2 and fig. 1). On average, 45,000 or 58% of the airway macrophages were removed by BAL, *i.e.* in the lavaged animals 42% of the macrophages remained in the intrapulmonary conducting airways.
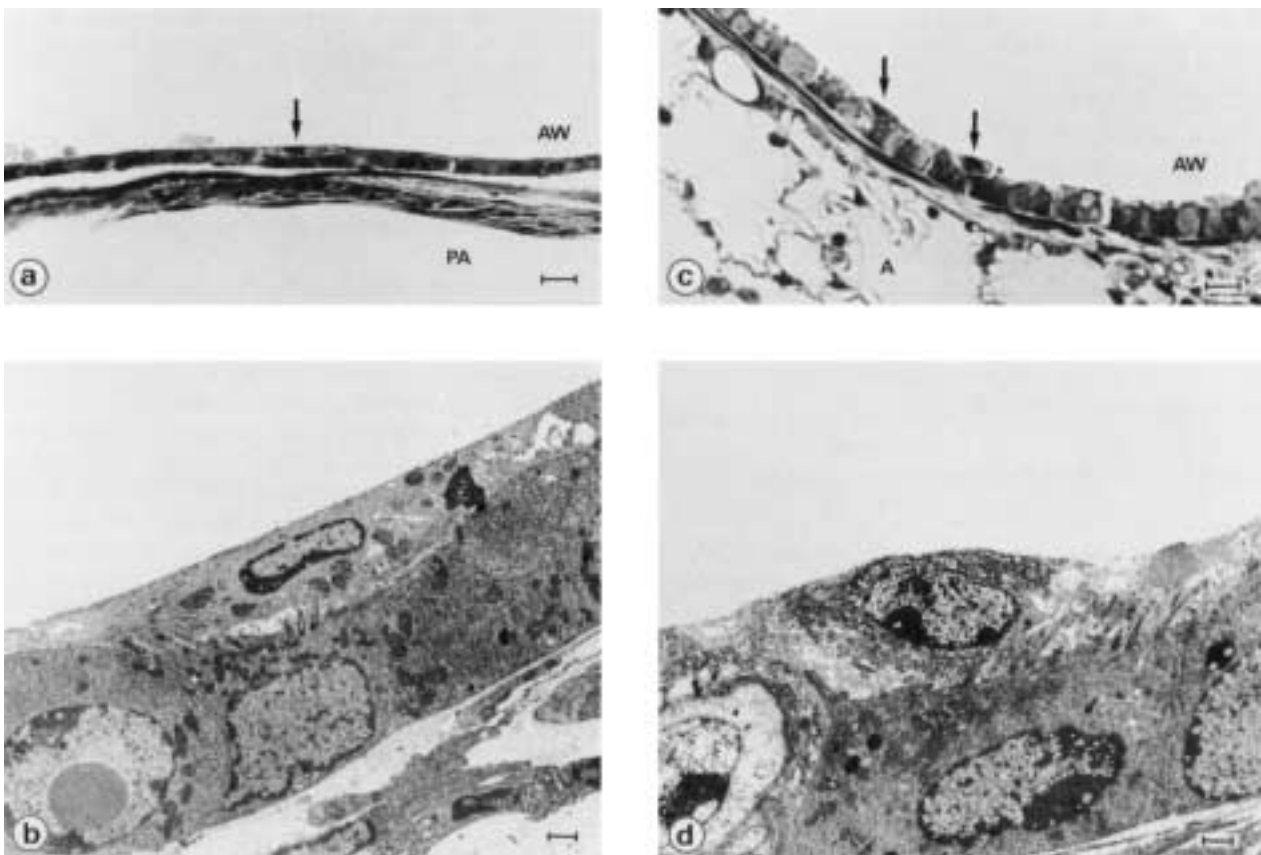

Fig. 2. – Macrophages (arrows) in large intrapulmonary conducting airways of control animals are spread out (a and b), whereas those of lavaged hamsters were less flattened (c and d). a and c) Light micrographs, Goldner staining, internal scale bar = 10 µm. b and d) Transmission electron micrographs, uranyl acetate and lead citrate staining, internal scale bar = 1 µm. AW: airway lumen; PA: pulmonary artery; A: alveolar space.
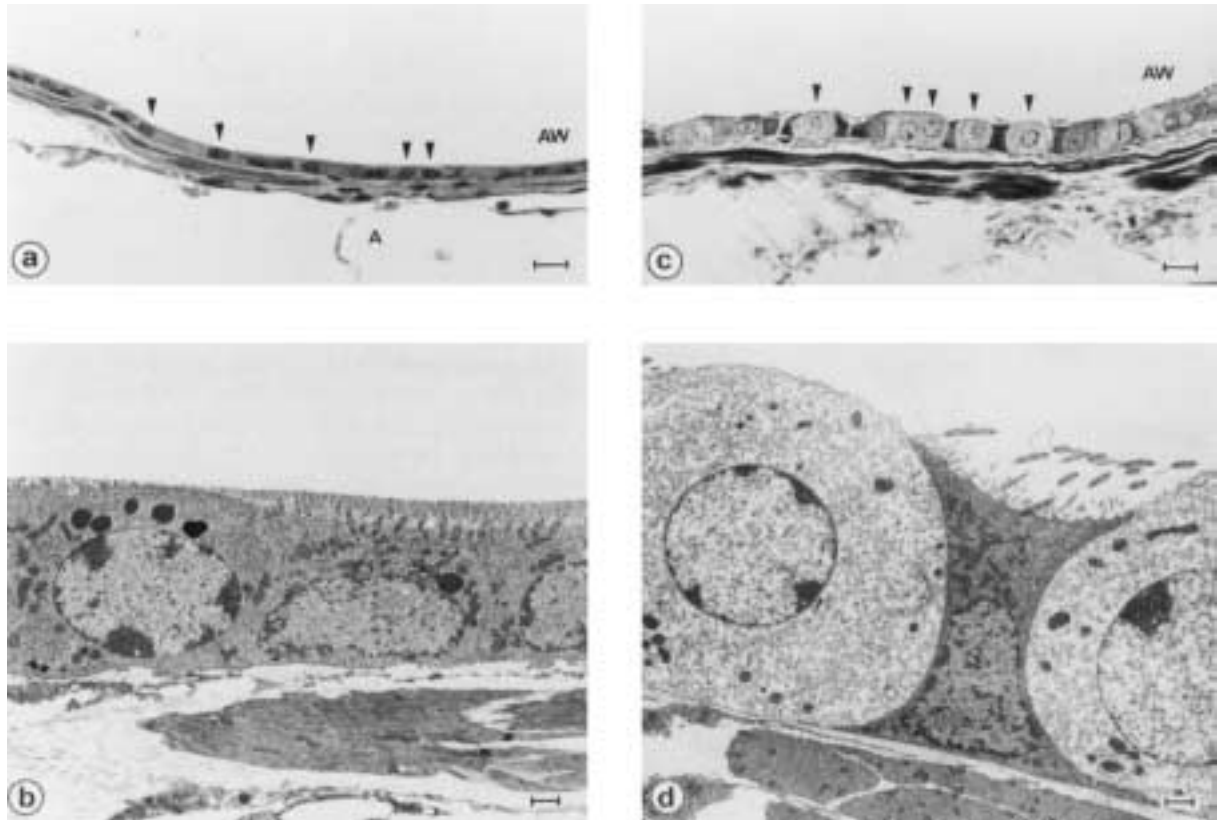
Fig. 3. – Airway epithelium of large intrapulmonary conducting airways of control animals (a and b), and of lavaged hamsters (c and d). The Clara cells (arrow heads) appear "bloated" after bronchoalveolar lavage (BAL). a and c) Light micrographs, Goldner staining, internal scale bar = 10 µm. b and d) Transmission electron micrographs, uranyl acetate and lead citrate staining, internal scale bar = 1 µm. AW: airway lumen; A: alveoli.

The number of 45,000 airway macrophages, revealed from the mean values of the stereologically estimated number of airway macrophages (mean est$N$(M), table 2) in Control group and BAL group animals (77,000 and 32,000, respectively), represents an approximate fraction of 5% of the number of all macrophages harvested by BAL (990,000, mean N(MBAL), table 1).

The estimated error due to stereology, CE^{2}est(N), was much higher in the animals of the BAL group than in the Control group (table 2).

Qualitative analysis of macrophages and epithelial cells

The lungs of all animals in the BAL group showed structural changes upon lavage. Airway macrophages of the Control group were spread out and in close contact with the airway epithelium, whereas those of the BAL group were less flattened (fig. 2). Occasionally, macrophages completely detached from the epithelium were found in the airways of BAL group animals.

The airway epithelial cells were also found to be altered after BAL. The Clara cells of the BAL group were bloated, squeezing the ciliated cells (fig. 3).

Discussion

This study showed that the number of macrophages in the intrapulmonary conducting airways of hamsters was 2.4 fold smaller after BAL than in control animals whose lungs had not been lavaged. However, in the four lavaged animals studied, on average, 42% of the total number of intrapulmonary airway macrophages were still found after BAL.

Several reasons for the relative ineffectiveness of BAL to remove airway macrophages have to be considered. Apart from the lavaging of the lungs in our study with cold wash fluid, all factors reported to influence the number of macrophages recovered by BAL [17, 18] were considered in our experimental procedure. The number of 0.99×10^6 (sd 0.59×10^6) macrophages recovered by BAL in our study corresponds to the 1.3×10^6 macrophages reported for hamsters by WARHEIT and HARTSKY [19]; there is no evidence that lavage was not successfully performed in our study.

We have no indication of significant macrophage displacement from alveoli to airways, since we very rarely found macrophages in the airway lumen; the macrophages that we found in the airways of BAL group animals did not have the typical round shape and were not filled with phagocytosed surfactant, as were the macrophages that we found in the alveoli.

We, therefore, suggest that there exists a subpopulation of airway macrophages that resists lavage. They might be those found in close contact with the epithelium [4, 6], of which at least a proportion is active in phagocytosis of particles deposited in airways, and is,

therefore, important for the clearance of particles from this lung compartment. Airway macrophages recovered by BAL might only represent a population of less adhesive macrophages, which might be functionally different from those more resistant to lavage. Further *in situ* studies are required to characterize subpopulations of airway macrophages with respect to their function and activity.

In the present study, about 5% of all the macrophages in the BAL fluid were estimated to be airway macrophages. This corresponds to the 5–8% which were reported for the rat [8].

The estimated mean coefficient of error, mean CE^2 (est$N$), for macrophages was much higher in animals of the BAL group than in the Control group. The reason for this difference might be that the macrophages in the control animals were more evenly distributed than in the hamsters where macrophages were removed by lavage. Yet, Equation (3) used to predict CE^2(est$N$) does not include the local variation or "nugget effect" within disectors [20, 21]. An improved formula including this effect is not yet available.

Structural changes of lavaged lungs, including intracellular oedema have been reported in dog lungs [22]. Furthermore, the blunted Clara cells that we have observed in the lavaged lungs point to alterations of cell membrane permeability, as was reported by BRIGHTWELL *et al*. [23], who detected marker enzymes for increased cell membrane permeability in small quantities in BAL fluid.

The loss of pulmonary surfactant by BAL leads to a collapse of the alveoli [24]. This might explain the significant difference of the mean lung volume between the BAL group and the Control group in our study (table 1), even though the final wash was performed with 2% BLES. Probably, it did not reach into all alveoli. However, the difference in lung volumes of the two groups does not affect our results, since the estimation of the total number of macrophages in a bounded reference solid with the fractionator is direct: there is no need to know the reference volume nor are any other measurements, *e.g.* section thickness, required.

The lavage technique used in this study on small experimental animals is an intensive whole lung lavage with large volumes of fluid, gentle massage of the lungs during each wash and a dwell-time of 7–10 min. This experimental method is clearly different from the segmental lavage techniques applied in clinical investigations in humans. The conclusions of the presented study, therefore, should be translated to the clinical situation with caution.

In summary, this *in situ* study in hamsters revealed: 1) that 42% of the conducting airway macrophages were not removed by bronchoalveolar lavage; 2) that about 5% of all macrophages in the bronchoalveolar lavage fluid were airway macrophages; and 3) that ultrastructural alterations occur in the airway epithelium after lavage. Apparently, there exists a population of airway macrophages that resists lavage by a yet unexplained enhanced adhesiveness to the airway epithelium. They might be functionally and morphologically different from those airway macrophages which are easily removed by lavage. Further studies are required to search for

markers which will allow these macrophage subpopulations to be distinguished. This could lead to a more profound understanding of airway macrophages and the mechanisms of clearance in the conducting airways.

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