Isolation of rat trachea interstitial fluid and demonstration of local cytokine production in lipopolysaccharide-induced systemic inflammation

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Semaeva E, Tenstad O, Bletsa A, Gjerde EB, Wiig H. Isolation of rat trachea interstitial fluid and demonstration of local cytokine production in lipopolysaccharide-induced systemic inflammation. J Appl Physiol 104: 809–820, 2008. First published January 10, 2008; doi:10.1152/japplphysiol.00846.2007.—Access to interstitial fluid from trachea is important for understanding tracheal microcirculation and pathophysiology. We tested whether a centrifugation method could be applied to isolate this fluid in rats by exposing excised trachea to G forces up to 609 g. The ratio between the concentration of the equilibrated extracellular tracer 51Cr-labeled EDTA in fluid isolated at 239 g and plasma averaged 0.94 ± 0.03 (n = 14), suggesting that contamination from the intracellular fluid phase was negligible. The protein pattern of the isolated fluid resembled plasma closely and had a protein concentration 83% of that in plasma. The colloid osmotic pressure in the centrifugate in controls (n = 5) was 18.8 ± 0.6 mmHg with a corresponding pressure in plasma of 22 ± 1.5 mmHg, whereas after overhydration (n = 5) these pressures fell to 9.8 ± 0.4 and 11.9 ± 0.4 mmHg, respectively. We measured inflammatory cytokine concentration in serum, interstitial fluid, and bronchoalveolar lavage fluid in LPS-induced inflammation. In control animals, low levels of IL-1β, IL-6, and TNF-α in serum, trachea interstitial fluid, and bronchoalveolar lavage fluid were detected. LPS resulted in a significantly higher concentration in IL-1β and IL-6 in interstitial fluid than in serum, showing a local production. To conclude, we have shown that interstitial fluid can be isolated from trachea by centrifugation and that trachea interstitial fluid has a high protein concentration and colloid osmotic pressure relative to plasma. Trachea interstitial fluid may also reflect lower airways and thus be of importance for understanding, e.g., inflammatory-induced airway obstruction.

interstitium; capillary filtration; extracellular matrix

SIGNIFICANT INFORMATION ON fluid exchange and transport between the blood and tissue compartments may be gained from analysis of interstitial fluid, i.e., the fluid portion of the extracellular compartment. As an example, will proteins dissolved in the interstitial fluid exert a colloid osmotic pressure (COP) that opposes the corresponding pressure in plasma in such a manner that changes in protein content may impair the normally well-functioning and autoregulated fluid balance (4).

In the airways, knowledge of the factors contributing to fluid homeostasis such as interstitial fluid COP is of vital importance because an edema in this organ may result in increased airway resistance and dyspnea (37, 44). Accordingly, access to interstitial fluid from the tracheobronchial tree is of major importance and may have therapeutical implications. In this context, fluid from trachea may be considered as a surrogate for lower airways. To our knowledge, only one study has dealt directly with the question of trachea interstitial fluid isolation before. In rabbits, Nordin et al. (23) divided and clamped the trachea to measure transvascular fluid exchange parameters. During this process, the tissue became edematous, and the authors were able to sample interstitial fluid and lymph. Several authors have addressed questions in relation to mechanisms behind fluid extravasation in the airways (for review, see Refs. 27, 35, 36), and the subepithelial (i.e., interstitial fluid) pressure that drives the inflammatory transudation into airway lumen has been estimated (30). Furthermore, Persson et al. (27) pointed to the importance of developing good in vivo models to study such phenomena. There are, however, no studies on native interstitial fluid isolation in control conditions and/or in smaller animals.

Although various techniques have been developed for tissue fluid isolation (for review, see Ref. 4), none of these seems suitable for trachea except sampling of lymph. In preliminary experiments with control rats, we were not able to discern lymphatics in trachea that we could cannulate and therefore had to use an alternative approach. In a recent study, our group (38) validated a centrifugation method for isolation of interstitial fluid in tumors and skin and later showed that this method is also applicable for this purpose in other tissues, such as bone marrow (39) and dental pulp (6), where the interstitium is difficult to access. Our major aim was to isolate interstitial fluid from the airways. Encouraged by our previous studies, we asked the question of whether centrifugation would be a suitable method to isolate tissue fluid from rat trachea, which is itself of interest but which may also serve as a surrogate for lower airways. If so, we asked whether the isolated fluid represented undisturbed interstitial fluid and whether a systemic inflammatory response was reflected in the interstitium of trachea in rats.

MATERIALS AND METHODS

Experimental Animals

The experiments were performed in Wistar rats (n = 87) of either sex (range 195–260 g, median 230 g). The rats had free access to food (a standard laboratory diet) and water before any experimental procedure. The rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). While anesthetized, we maintained the rats’ body temperature at 37 ± 1°C using a heating pad and lamp. Polyethylene (PE-50) catheters were placed in the femoral vein for injection of tracers and substances (see below) and in the femoral artery for blood sampling. Blood was sampled in heparinized vials for isolation of plasma unless otherwise specified. Rats were killed by cardiac arrest induced under anesthesia with an intravenous injec-

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tion of saturated potassium chloride. All animal experiments were conducted in accordance with regulations of the Norwegian State Commission and with preapproval from the ethical committee of University of Bergen.

Centrifugation Technique

In our attempt to isolate interstitial fluid from the trachea, we used a modification of the method described by Wiig et al. (38) in tumors and skin. The euthanized rats were transferred immediately after cardiac arrest to an incubator at room temperature and 100% relative humidity to avoid evaporation from the trachea. The trachea was rapidly dissected free of surrounding tissue, excised, and split longitudinally. The split trachea was placed with the serosa side facing downward on a nylon mesh basket (pore size of \( \sim 15 \times 20 \) μm), which was placed in an Eppendorf centrifugation tube so that the sample was kept up from the bottom of the tube (3). The preweighed tube with nylon basket was capped immediately and reweighed after we added the trachea sample before centrifugation for 10 min in an Eppendorf 5417R centrifuge placed in a cold room at 4°C. Immediately after centrifugation, the tube was brought back into the incubator.

Trachea interstitial fluid that accumulated at the bottom was collected in glass capillaries, which were either closed immediately for later analysis or diluted in buffer for HPLC (see below). Typical samples were 0.5–2 μl.

To reduce the risk of compression of cellular elements of the trachea, the centrifugation speed was as low as possible to collect an interstitial fluid sample. In initial experiments, the samples were spun at 424 g (2,000 rpm) for 10 min. In subsequent experiments, we reduced the G force successively in 100-rpm steps to explore the minimal G force needed to produce a sample. We found that it was always possible to isolate fluid at 239 g (1,500 rpm). Generally, with increasing speed of centrifugation, an increased amount of erythrocytes accumulated in the centrifugate. Exposing the tracheal tissue to G forces of 609 g (2,500 rpm) and above resulted in progressive hemolysis of the centrifugate. Therefore, 239 g (1,500 rpm) was used for sampling of trachea fluid unless otherwise specified. In this study, this fluid is called trachea interstitial fluid.

Validation Experiments

Assessment of potential admixture of intracellular fluid. Our aim was to isolate native, uncontaminated interstitial fluid from trachea. To estimate any contribution of cellular fluid, we studied the concentration ratio of an extracellular tracer in trachea interstitial fluid and plasma collected from rats \( (n = 20) \). Addition of cellular fluid not containing tracer will appear as a reduced concentration in centrifugate relative to plasma. After anesthesia and catheter placement, the animals were bilaterally nephrectomized to prevent excretion of the extracellular tracer. Volume of the catheter in the femoral vein, the animals were bilaterally nephrectomized to prevent excretion of the extracellular tracer. After a regular tracer equilibration period of 120 and 5 min, respectively, the experiment was terminated. The trachea was isolated and incised as described above, and the cut proximal and distal ends of the trachea were wiped carefully with blotting paper to avoid potential contamination from severed blood vessels. To avoid the use of an absorbing material, which may disturb the epithelial barrier function and sample subepithelial (i.e., interstitial) fluid and solutes (10), we decided to try an alternative approach. With the assumption that some trachea surface liquid would adhere to a nonabsorbing catheter surface, a preweighed PE-50 catheter was carefully stroked along the inner circumference of the trachea. Attempt was made to avoid contact between the catheter and the exposed surface of the trachea wall. The catheter was transferred to a preweighed tared vial and reweighed. After 40 μl of HPLC buffer were added, the sample was counted together with plasma and trachea. After counting was completed, the trachea surface liquid protein distribution pattern was determined by HPLC and compared with that of trachea interstitial fluid and plasma.

In another series of experiments \( (n = 5) \), we examined the transport of substances from trachea surface liquid through the interstitium into the centrifugate to determine the possible contribution of surface liquid macromolecules to isolated interstitial fluid. In these experiments, 1 μl of a tracer mixture containing stock solution of 51Cr-EDTA (5 \( \times \) 10-4 MBq) and 125I-HSA (5 \( \times \) 10-7 MBq) was carefully deposited onto the trachea surface. The trachea was transferred to a centrifuge tube for centrifugation at 239 g, and isolated interstitial fluid and trachea were counted as described above.

Estimation of volume of trachea surface liquid. To calculate the mucosa surface area of trachea and thus be able to estimate the volume of the trachea surface liquid, we determined the inner diameter in proximal and distal end of excised tracheas \( (n = 6) \) in anteroposterior and frontal directions using a stereomicroscope equipped with a graticule.

Fluid Volumes

In a separate series of experiments \( (n = 6) \), we determined the extracellular fluid and plasma distribution volumes in trachea utilizing 51Cr-EDTA and 125I-HSA, respectively. After anesthesia and placement of the catheter in the femoral vein, the animals were bilaterally nephrectomized to prevent excretion of the extracellular tracer. Volumes were determined with 51Cr-EDTA and 125I-HSA as described above, except that the dose of 51Cr-EDTA was 0.05 MBq. Tissue samples taken from the trachea were placed in tared, covered vials and weighed and counted as described above. Plasma equivalent distribution volumes \( (V) \) for 51Cr-EDTA and 125I-HSA were calculated as follows: \( V = (cpm/g tissue)/(cpm/ml plasma) \).
HPLC of Plasma and Fluid Isolated From Trachea

We wanted to compare trachea interstitial fluid with that eluted into a buffer to investigate whether the centrifugation process per se affected the composition of the interstitial fluid. For this purpose, trachea was soaked in PBS containing 0.02% azide for 2 h, and trachea eluate was isolated. Furthermore, to induce a situation with cellular damage and release of cellular proteins to the surrounding solution, we isolated extracts from trachea after induction of cell damage. These tracheas (n = 5) were freeze dried five times and crushed before being soaked in PBS for 24 h. After centrifugation, the supernatant was collected for further analysis.

The distributions of macromolecules in isolated trachea interstitial fluid, eluate, tissue extracts (homogenates), trachea surface liquid, and plasma were determined by high-resolution size-exclusion chromatography using three 7.8 mm (ID) × 30.0 cm TSKgel G3000SWXL or two 4.6 mm × 30 cm SuperSW3000 columns coupled in series (Tosoh Biosciences, Stuttgart, Germany) with an optimal separation range for globular proteins of 10–500 kDa.

The total protein concentration and the concentration of albumin and IgG in the eluted fluids were measured by UV detection at 220 nm on an Etan HPLC system (GE Healthcare), as described in previous publications (38, 40). Isolated fluid, 0.2–2 µl, was diluted in mobile phase (0.1 M Na2SO4 in 0.1 M phosphate buffer, pH 7.0) in a total volume of 40 µl and injected onto the HPLC system. The injected volumes were 10 µl (super SW3000 columns) or 35 µl (G3000SWXL columns) using 20- or 200-µl loops, respectively. The flow was held constant at 0.2 or 0.5 ml/min. Human IgM, α1-macroglobulin, fibrinogen, haptoglobin, rat IgG, BSA, and ovalbumin were purchased from Sigma-Aldrich and used as standards to calculate the range of hydrodynamic radii and apparent molecular weights of the protein fractions from plasma and trachea interstitial fluid.

Identification of Plasma Protein by HPLC-Mass Spectrometry

After HPLC separation as described above, macromolecular fractions from plasma and trachea interstitial fluid were exchanged into 100 mM ammonium bicarbonate and concentrated to a final volume of 25 µl using a Microcon YM-50 centriugal filter unit (Millipore). The samples were then denatured by addition of 25 µl trifluoroethanol (Sigma-Aldrich T-8132) and 2.5 µl diethyldithioctetan (Sigma-Aldrich D-5545) at 60°C for 45 min and digested at 37°C overnight by trypsin (Sigma-Aldrich T6763, protease-to-protein ratio of 1:20) in accordance with a protocol provided by Agilent Technologies (http://www.chem.agilent.com; publication no. USHUPO3). Digested proteins from plasma and trachea interstitial fluid were desalted by C18 peptide cleanup spin tubes (Agilent Technologies) and analyzed with an Agilent 1100 LC/MSD Trap XCT Plus system consisting of a nanoflow pump, wellplate sampler, capillary pump, HPLC-Chip/MS interface, and a Trap XCT Plus mass spectrometer. The HPLC-Chip contained a 0.075 × 43 mm ZORBAX 300SB C18 5-µm column and an integrated 40-µl enrichment column packed with the same material (part no. G4240-62001). Processing of the MS/MS data was performed with Spectrum Mill MS proteomics workbench software (Rev A.03.02.060) using identity mode and default settings.

Colloid Osmotic Pressure

The COP levels in trachea interstitial fluid and in plasma were measured in a colloid osmometer designed for submicroliter samples (41), using membranes with a cutoff size of 30 kDa. Usually, samples of 0.5–1 µl were applied to the osmometer membrane.

Analysis of Inflammatory Mediators in Serum and Interstitial Fluid

To be able to monitor whether an inflammatory process was reflected in the interstitial fluid, the proinflammatory mediators IL-β, IL-6, and TNF-α and the anti-inflammatory mediator IL-10 were measured simultaneously in samples using Lincoplex kit (Linco Research, St. Charles, MO) according to the manufacturer’s instructions. In these experiments, analyses were performed on serum as recommended by the manufacturer. The samples were prepared for analysis by dilution with serum matrix diluent provided with the kit. Serum and interstitial fluid samples were reacted with a mixture of fluorescent beads bound with specific anticytokine primary antibodies, resulting in binding of the cytokines in the sample to the beads with the corresponding antibody. Then, biotin anticytokine secondary antibodies were added and allowed to bind to the cytokine-bead complex, followed by addition of fluorescent phycoerythrin-conjugated streptavidin. Total surface fluorescence was then measured with a BioPlex fluorescent flow-based fluorescence detection system (Bio-Plex multiplex suspension system; Bio-Rad). A broad range of standards (4.8–20,000 pg/ml) was provided in the multiplex kit, and the multiplexed assay was analyzed on a flow cytometer (Luminex100, Luminex). The minimum detection level ranged from 2.3 (IL-1β) to 9.8 (IL-6) pg/ml. To prevent potential protein degradation of mediators present in low concentrations, we added protease inhibitor cocktail (P8340, Sigma) to these samples. The stock solution was diluted 1:10 in 0.9% saline, and 1 µl of the diluted solution was added to the tubes before centrifugation for an immediate effect, whereas undiluted protease inhibitor (15 µl/ml) was added to serum. After interstitial fluid isolation, the samples were frozen immediately and stored at −80°C until further analyses were performed.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) is a versatile and much used technique to study, e.g., signaling substances liberated from the airways. Fluid sampled by BAL will mainly represent airways distally to trachea and reflect more closely the surface liquid than the present centrifugation technique. To compare the composition of the interstitial fluid isolated by centrifugation from trachea to that obtained in more distal airways by BAL method, we added a series of experiments in which lavage fluid was sampled. In these experiments, PBS totaling 9 ml was instilled into trachea via a tracheal cannula, in three 3-ml aliquots. Around 5–7 ml of BAL fluid were recovered for each rat. Protease inhibitor (15 µl/ml) was added immediately after sampling, and the samples were stored at −80°C until analysis.

Immunohistochemistry

Tracheas from rats exposed to LPS for 3 h (n = 2) and from control rats (3 h vehicle) (n = 2) were removed and fixed in 4% paraformaldehyde with 0.2% picric acid. The tissue was then rinsed in 0.1 M phosphate buffer, soaked in 30% sucrose overnight, and stored at −80°C until they were sectioned. The specimens were then embedded in Tissue-Tek OCT compound (Sakura, Zoeterwoude, The Netherlands), and 10-µm-thick sagittal sections were cut in a freezing (−20°C) slide microtome. The immunoreactions were performed on precoated glass slides (SuperFrost Plus, MenzelGlaser, Braunschweig, Germany). For the immunohistochemical staining of possibly cytokine-producing cells, the following antibodies were used: mouse anti-rat W3/13 (dilution 1:400; AbD-Serotec, Oxford, UK), which stains all leukocytes except B lymphocytes; and mouse anti-rat ED1 (dilution 1:400; AbD-Serotec), which stains tissue macrophages. For the cytokine localization, we used rabbit anti-rat IL-1β (dilution 1:150; Santa Cruz Biotechnology, Santa Cruz, CA). Alternate serial sections were double stained with IL-1β and W3/13 or ED1 antibodies. Sections were blocked with 5% normal goat serum (Chemicon) before incubation with primary antibodies (overnight at 4°C). After sections were washed, samples were incubated with corresponding secondary antibodies Cy3 goat anti-rabbit IgG (dilution 1:300; Jackson ImmunoResearch, West Grove, PA) and Cy2 goat anti-mouse IgG (dilution 1:300; Jackson ImmunoResearch) for 1 h at room temperature. The sections were coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) with 4′,6-diamidino-2-phenylindole, which stains DNA, and viewed with a fluorescence
microscope (Axio Imager, Carl Zeiss MicroImaging). The images were captured with AxioCam MRm camera (Carl Zeiss), and the AxioVision 4.4 (Carl Zeiss) imaging system was used for analyses.

The specificity of the immune reaction was tested by omission of the primary antibodies or substitution with isotype controls.

Experimental Protocol

**LPS-induced acute inflammation.** To study whether a condition known to induce respiratory symptoms and respiratory distress syndrome resulted in changes in inflammatory mediators in trachea interstitial fluid, we induced a systemic inflammation by infusion of LPS from *Escherichia coli* (serotype 0127:B8; Sigma). The LPS was diluted in PBS containing 0.1% BSA, and the stock solution had a concentration of 0.25 mg/ml. The rats received a dose of 3.0 mg/kg LPS by infusion of stock solution intravenously and were observed for 3 h (n = 10). In these experiments, the femoral artery was catheterized for monitoring arterial pressure. Five of these rats were used for isolation of interstitial fluid by centrifugation; the others were subjected to BAL. In this series, protease inhibitor was added to trachea and BAL fluid and serum as described above. Samples were stored at −80°C until analysis.

To investigate whether leukocytes migrated to trachea interstitial fluid during LPS treatment, we made a smear of isolated interstitial fluid in LPS-treated (n = 2) and control (n = 2) rats after dilution in 10 μl PBS. All smears were stained with Colorrapid (Lucerna-Chem, Luzern, Switzerland). To study whether the LPS treatment influenced the fluid volumes in trachea, we measured extracellular fluid and plasma volumes using 51Cr-EDTA and 125I-HSA as described above (see Fluid Volumes above) (n = 6).

**Overhydration.** We asked whether a volume load known to induce a dilution of plasma proteins and tissue overhydration was reflected in trachea interstitial fluid. To this end, a volume of Ringer solution corresponding to 15% of body weight was infused intravenously with an infusion pump over a period 30 min in five rats. After infusion, an equilibration period of 1 h was allowed. After rats were killed, interstitial fluid from trachea was isolated, and COP levels in interstitial fluid and plasma were measured as describe above.

Statistical Methods

All values are means ± SE unless otherwise stated. Differences were tested with two-tailed t-test using paired comparison when appropriate or ANOVA followed by Dunn or Bonferroni test. P < 0.05 was considered statistically significant.

RESULTS

The weight of an isolated trachea used in our experiments averaged 0.047 ± 0.01 g (n = 22). Typical trachea fluid samples were straw colored. Occasionally, some erythrocytes were collected at the bottom of the centrifugation tube, but these were avoided on removal of the sample from the tube and were not included in further analyses. Smears (n = 2) showed that the isolated fluid was practically cell free in the control situation. The amount isolated at the lowest force reported here (239 g) was typically 1–1.5 μl, increasing to 1.5–2 μl at 424 g. If we assume a density of 1.0 g/ml, these volumes suggest 2–4% of the tissue wet weight was removed by the tissue centrifugation. There was no clear relationship between rat body weight and thus trachea weight and the amount of interstitial fluid that was isolated by centrifugation.

Analyses of Trachea Fluid

To determine whether the interstitial fluid was concentrated or diluted during the isolation process, we measured recovered tracer that had equilibrated in extracellular fluid (51Cr-EDTA) or distributed in plasma (125I-HSA), and the results are shown in Fig. 1.

The average concentration of 51Cr-EDTA in trachea fluid relative to that in the plasma was 0.94 ± 0.03 (n = 14), 0.94 ± 0.03 (n = 12), and 0.49 ± 0.04 (n = 3) at centrifugation speeds of 239, 424, and 609 g, respectively (Fig. 1A). Although the ratios found at the two lowest G forces were close to unity, both differed significantly from 1.0 (P < 0.05 for both ratios).

We also determined the amount of fluid in the centrifugation that derived from plasma and the recovery in the centrifugate of the intravascular tracer 125I-HSA. The ratios for 125I-HSA corresponding to those of 51Cr-EDTA above was 0.017 ± 0.004 (n = 11), 0.032 ± 0.008 (n = 8), and 0.058 ± 0.017 (n = 3) at the centrifugation speeds of 239, 424, and 609 g, respectively (Fig. 1B).

By careful circumferential stroking of inner surface of the trachea with a plastic catheter, we were able to isolate 0.2–0.4 mg (corresponding to 0.2–0.4 μl assuming a density of 1.0 g/ml) of surface liquid from an isolated trachea.

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**Fig. 1. Ratio of trachea fluid-to-plasma concentration of an extracellular and intravascular tracer as a function of applied G force: A: an extracellular tracer (51Cr-labeled EDTA) was equilibrated in extracellular fluid isolated by centrifugation (centrif). Increasing the G force resulted in a reduced trachea fluid-to-plasma ratio of the extracellular tracer, indicating dilution of the centrifugate. All ratios differed significantly from 1.0. B: an intravascular tracer (125I-labeled human serum albumin (125I-HSA)) had equilibrated for 5 min before fluid isolation. The trachea fluid-to-plasma ratio reflects plasma contribution to the centrifugate. Values are means ± SE; n = 14 rats for 239 g, n = 12 rats for 424 g, and n = 3 rats for 609 g. ANOVA: *P < 0.05 compared with the ratio at 239 g.**
When analyzing for tracer recovery in this fluid after 120 and 5 min of equilibration, we found that the mean concentration of $^{51}$Cr-EDTA and $^{125}$I-HSA was 0.69 ± 0.05 ($n = 5$) and 0.004 ± 0.001 ($n = 5$) relative to that of plasma, respectively.

When a mixture of $^{51}$Cr-EDTA and $^{125}$I-HSA was applied to the mucosa ($n = 5$), we were on average able to recover 0.019 ± 0.011 and 0.011 ± 0.004 of the dose of tracer, respectively.

The inner diameter (estimated as mean value of proximal and distal diameter) of trachea ($n = 6$) averaged 1.8 ± 0.1 and 2.3 ± 0.1 mm in anteroposterior and frontal direction, respectively, and the mean length of the trachea used for centrifugation was 30.0 ± 0.2 mm. These numbers were used to calculate a mean inner trachea surface area of samples used in our experiments of 194 mm$^2$ and were found as the product of the circumference ($C$) of an ellipse [calculated as $C = \pi \times \sqrt{2 \times (a^2 + b^2)}$] and trachea length.

**Fluid Distribution Volumes in Trachea**

We quantified the space of origin of the extracellular fluid isolated from trachea. Total extracellular fluid volume, calculated as 120-min distribution volume of $^{51}$Cr-EDTA, was 0.38 ± 0.02 ml/g wet wt ($n = 8$). The intravascular plasma volume determined as the 5-min distribution volume of $^{125}$I-HSA was 0.027 ± 0.006 ml/g wet wt. Thus the trachea interstitial volume, calculated as the difference between extracellular fluid and plasma volume, was 0.35 ± 0.03 ml/mg wet wt.

LPS exposure did not result in any changes in the fluid distribution volumes in trachea. After exposure, the total extracellular fluid volume was 0.37 ± 0.02 ml/g wet wt, with a corresponding intravascular plasma volume of 0.023 ml/g wet wt.

**Protein Patterns in Trachea Fluid Analyzed With HPLC**

In an attempt to validate that the fluid isolated by centrifugation was of extracellular origin, we subjected the samples from six rats to HPLC analysis and compared the patterns of isolated fluids with those of corresponding plasma. Representative patterns of plasma, of trachea fluid isolated by centrifugation, of eluate from intact trachea eluted in PBS for 2 h, and of eluate from crushed trachea eluted in PBS for 24 h are shown in Fig. 2.

The protein peaks found in plasma were also found in trachea centrifugate and eluate (Fig. 2, A–C) ($n = 6$), whereas the pherograms from crushed trachea samples ($n = 5$) differed substantially (Fig. 2D), indicating significant intracellular fluid contamination in the latter.

For molecules smaller than albumin, the elution pattern of plasma, trachea interstitial fluid, and eluate differed slightly. Whereas plasma had practically no peaks eluting after albumin (Fig. 2A), trachea interstitial fluid contained minor peaks representing proteins not present in plasma (Fig. 2B). These peaks were more pronounced in eluate (Fig. 2C), indicating some addition of intracellular fluid and/or fluid from the surface layer (see below).

To compare the relative distribution of differently sized macromolecules in plasma, trachea interstitial, and surface liquid, we normalized all pherograms with respect to albumin ($n = 5$) and plotted the elution curves for interstitial and surface liquid together with that of plasma (Fig. 3). Plasma and trachea interstitial fluid from four rats were also analyzed by on-line reverse-phase nano liquid chromatography coupled with ion-trap mass spectrometry to establish the identity of the most abundant proteins derived from a sample corresponding to ~20 nl of undiluted fluid (Table 1). Although 20–30 of the most abundant plasma proteins were also found in trachea fluid, the relative abundance of proteins was different.
centrifugate, this fluid also contained intracellular enzymes and proteins known to reside in or on the epithelial cell layer. The contribution of intracellular proteins was most pronounced in fractions 7 and 9 (Fig. 3A). When comparing plasma and interstitial fluid, we observed that plasma contained a significantly higher fraction of proteins from high-molecular-mass fractions 2–6, as shown in Table 1 and exemplified in Fig. 3A, suggesting a certain size selectivity of the traheal capillaries for these proteins. Of note, the first peak in pherograms from traheal interstitial fluid clearly exceeded that in plasma (interstitial fluid-to-plasma protein concentration ratio of ~7; Table 1), indicating local production of IgM. We also observed that peak 7 in the pherogram from traheal interstitial fluid did not superimpose completely with the corresponding peak in plasma (Fig. 3), resulting in an interstitial fluid-to-plasma protein concentration ratio slightly greater than 1 and reflecting minor contamination from epithelial-derived proteins (Table 1). A somewhat different picture was found when we compared plasma and traheal surface liquid (Fig. 3B). Whereas the total protein concentration was low (Fig. 4), surface liquid contained additional high-molecular-mass peaks and a significant amount of proteins eluting in the low-molecular-mass range (≤60 kDa) that were not found in plasma (Fig. 3B). Most of these proteins probably originate from shed epithelial cells and mucus.

The results from the HPLC determination of albumin and total protein in traheal interstitial and surface liquid and plasma (n = 6 for all) are shown in Fig. 4. In traheal interstitial fluid, the concentration of total protein and albumin relative to that in plasma averaged 83 and 86%, respectively (P < 0.05 for both comparisons). The corresponding average numbers for traheal surface liquid were 14 and 10%.

**COP in Traheal Interstitial Fluid**

COP levels in fluid isolated from traheal in control situations (n = 5) and after overhydration of 15% of body weight (n = 5) are shown in Fig. 5. In the control situation, COP in traheal interstitial fluid averaged 18.8 mmHg with a corresponding pressure in plasma of 22 mmHg.

Overhydration induced by infusion of saline corresponding to 15% of body weight resulted in significant reduction in COP in plasma and in traheal interstitial fluid. Thus COP levels in fluid isolated from the traheal and plasma after an equilibration period of 60 min following the infusion averaged 9.8 and 11.9 mmHg, respectively. In control situations and after overhydration, the COP in traheal interstitial fluid was lower than the corresponding COP in plasma (P < 0.05, paired t-test).

**Cytokine Concentrations in Serum, Traheal Interstitial Fluid, and BAL Samples**

We asked whether a systemic inflammation resulted in detectable changes in inflammatory mediators in traheal interstitial fluid and whether there was a local production of cytokines in traheal. Also, we wanted to compare the present fluid isolation method to the more commonly used method for demonstration of potential inflammatory reactions in airway tissue, namely, the BAL method. It should be noted, however, that substances released to the lavage fluid will be diluted by the large lavage volume and that BAL fluid originates mainly from lower airways (see above). These facts should be kept in mind when interstitial fluid and lavage data are compared. In control animals, we found low levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in samples from serum, traheal interstitial fluid, and BAL fluid (Table 2), whereas the antiinflammatory cytokine IL-10 was not detectable in any of the fluids examined in this condition.

Induction of a systemic inflammatory response resulted in significant changes in all of the cytokines that were measured. In the LPS-treated animals, the levels of IL-1β, IL-6, IL-10, and TNF-α in serum, traheal interstitial fluid, and BAL samples were all increased compared with results shown in controls (Fig. 6). Of interest, we found that the average concentration of IL-1β was significantly higher in traheal interstitial fluid (4.4 ng/ml) than in serum and BAL samples (0.27 and 0.07 ng/ml, respectively). Furthermore, the level of IL-6 was
increased in trachea interstitial fluid, serum, and BAL samples, averaging 87.1, 46.3, and 1.4 ng/ml, respectively. Again, the increase in trachea interstitial fluid was significantly higher than that in serum, suggesting that there was a local production of these inflammatory cytokines in trachea in this condition. Of interest, the control-to-LPS ratio between the concentrations of the two proinflammatory cytokines produced locally (IL-1 and IL-6) was practically identical in trachea interstitial and BAL fluid (62.8 and 62.2, respectively), suggesting a similar dilution and thus response pattern for these two cytokines in upper and lower airways.

For TNF-α, another of the proinflammatory cytokines that were assayed, the expression pattern was somewhat different. Significantly higher mean concentrations of this cytokine were found in trachea interstitial fluid, serum, and BAL samples in LPS-treated animals (1.7, 4.5, and 1.3 ng/ml, respectively) than in controls. In contrast to

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<td>Haptoglobin (trimer)</td>
<td>200-300</td>
<td>5.1-5.6</td>
<td>0.65±0.33</td>
</tr>
</tbody>
</table>

Table 1. Identification of abundant proteins found in plasma and interstitial fluid

Values for IF/P are means ± SE; n = 6 rats. Peak numbers refer to corresponding numbers in Fig 3. Apparent molecular mass and Stokes radius were estimated by size exclusion chromatography. IF/P, interstitial fluid-to-plasma protein concentration ratios; GAPDH, glyceraldehyde 3-phosphatedehydrogenase; NA, not applicable. *Trachea fluid-specific protein.

Fig. 4. Concentration of total protein and albumin in plasma (solid bar), trachea interstitial fluid (IF; open bar), and trachea surface liquid (SL; hatched bar) estimated using HPLC (n = 6). Values are means ± SE. *P < 0.05 compared with corresponding plasma value.

Fig. 5. Colloid osmotic pressure in plasma (COPp) and trachea interstitial fluid (COPif) isolated by centrifugation at 239 g for 10 min in control situation (n = 5) and after overhydration (n = 5) induced by infusion of saline corresponding to 15% of body weight. Values are means ± 1 SE. *P < 0.05 compared with corresponding control value; §P < 0.05 compared with corresponding plasma value.
what was found for IL-1β and IL-6, the concentration of TNF-α in serum was significantly higher than in trachea interstitial fluid. Clearly, LPS treatment also resulted in an activation of the anti-inflammatory cytokine IL-10. The level of IL-10 in BAL samples was detectable, averaging 0.02 ng/ml. Somewhat higher mean levels of 0.36 ng/ml were found in trachea interstitial fluid, whereas a significantly higher mean concentration of this cytokine of 0.82 ng/ml was found in serum.

Smears made from trachea interstitial fluid showed that the fluid contained scattered granulocytes and mononuclear cells, but the number was insufficient for differential counting. A low cell count was to be expected because of the fluid isolation procedure and because a possible pellet was avoided during sample removal from the tube.

Immunostaining

Because the analysis of cytokines in the interstitial fluid suggested that some cytokines were locally produced, we aimed at verifying the results by immunostaining and possibly revealing the cellular source of cytokines. Therefore, we performed double staining with IL-1β and W3/13 or ED1. IL-1β was chosen because it was the most differentially expressed cytokine in trachea interstitial fluid and serum. Control trachea exhibited normal tissue architecture, whereas LPS exposure caused flattening of the epithelium and enlargement of the tracheal glands in the submucosa. There was some staining for IL-1β in the tracheal epithelial cells in the control rats; however, after LPS treatment, the staining for this cytokine was markedly increased in the lamina propria and submucosa (Fig. 7, B and F). Most of the cytokine-positive cells in these areas were also ED1 positive (Fig. 7, D and F) or W3/13 positive (data not shown), indicating that macrophages, T lymphocytes, and cells of the granulocyte lineage (most likely neutrophils) were producing IL-1β. In addition, epithelial cells and chondrocytes in the hyaline cartilage exhibited staining for IL-1β in the LPS-treated rats (Fig. 7, B and F), and they may both contribute together with cells in lamina propria and submucosa to the observed increase in local production of IL-1β after LPS challenge.

All of the negative controls showed a lack of specific immunostaining.
DISCUSSION

In the present study, we asked whether it was possible to isolate interstitial fluid from trachea and have shown that tissue fluid samples can be obtained by exposing isolated trachea tissue to increased G forces by centrifugation. We have evaluated the method with respect to potential contamination of fluid from cellular sources, as well as trachea surface fluid, and found that the isolated fluid is representative for tracheal interstitial fluid at G forces \( \leq 424 \) g. At higher G forces, a dilution from cellular fluid is likely to occur. Below, we will discuss the foundation of our conclusions and the implications for fluid balance studies in the trachea.

Evaluation of the Centrifugation Method

Although we were able to isolate some fluid from the trachea by centrifugation, the major question was to provide convincing evidence that this fluid derives from the extracellular fluid phase. By determining the ratio of the extracellular tracer \(^{51}\text{Cr}-\text{EDTA}\) in isolated trachea fluid and plasma, we could also determine the origin of the centrifuged fluid. Addition of intracellular fluid not containing \(^{51}\text{Cr}-\text{EDTA}\) would thus dilute the centrifugate and make the centrifugate-to-plasma ratio \(< 1.0\). In contrast, a centrifugate-to-plasma ratio of \(> 1.0\) would indicate that some fluid not containing tracer had been removed from the centrifugate, e.g., by cell swelling or evaporation. We
found a centrifugate-to-plasma ratio of 0.94 at 239 and at 424 
\(g\), with both ratios significantly different from 1.0. These data 
suggest that ~6% of the isolated fluid is of intracellular origin, 
possibly deriving from erythrocytes, connective tissue cells, 
and/or epithelial cells damaged during centrifugation. This 
assumption is supported by the finding of intracellular proteins 
(e.g., actin) in the interstitial fluid. Notwithstanding the fact 
that the passage of tracers applied to the trachea surface fluid 
to centrifugate was low, addition of surface fluid will add to the 
interstitial fluid dilution effect because such fluid was not fully 
equilibrated with respect to \(^{51}\)Cr-EDTA after 2 h of circulation 
time. We tried to minimize this problem by reducing the 
applied G force and optimizing the tissue handling, e.g., by 
rapid processing of the tissue to avoid degradation. Exceeding 
424 
\(g\) resulted in an even lower centrifugate-to-plasma ratio, 
indicating that an increased dilution occurred. This observation 
is probably a result of further extrusion of cellular fluid and/or 
cell damage and shows that a G force \(\leq 424 \ g\) should be used 
for tracheal fluid isolation, in agreement with previous data for 
skin and tumors (38).

It is possible that trachea surface fluid may be part of the 
fluid sampled by centrifugation, and we thus attempted to 
quantify surface fluid admixture. There are several reasons for 
us to conclude that such admixture or contamination is negligible. 
First, the total volume of trachea surface fluid is small 
compared with the interstitial fluid volume. The average 
trachea sample weight was 47 mg, and with an average extracel-


\(\text{\textit{m}}\) larular fluid fraction of 38% this results in a total extracellular 
fluid volume in our trachea samples of 17.9 \(\mu\text{m}\) assuming a 
density of 1.0 mg/\(\mu\text{m}\). We estimated that the area of the inner 
surface of our trachea sample averaged 194 mm\(^2\). The thick-


\(\text{\textit{m}}\) ness of the surface layer is 5–10 \(\mu\text{m}\) (45), suggesting a surface 
fluid volume of 10–20 \(\mu\text{m}\), i.e., ~10% of the extracellular fluid 
volume. Second, we measured the passage of tracer from 
surface fluid to centrifugate and found a very low recovery 
(e.g., 1% of the applied dose of HSA). In addition, we esti-
mated the total protein, albumin, and IgG concentrations in 
trachea surface fluid and found these to be 10–15% of the 
concentration in plasma. Thus a combined low-volume fraction 
compared with total interstitial fluid, low passage of albumin to 
the centrifugate, and low protein concentration will result in a 
negligible amount of trachea surface fluid and proteins in the 
fluid isolated by centrifugation. Our observed albumin concen-
tration in trachea surface fluid of ~10% that in plasma is 
higher than that found in humans (16), a difference that may be 
due to species differences and experimental conditions.

If we accept that the pattern for tissue eluate is representative 
for extracellular fluid, the comparison of the pherograms of 
centrifugate and eluate suggests that the addition of possible 
intracellular proteins is very low. To show the sensitivity of our 
approach to detect intracellular protein contamination to the 
centrifugate, we made extracts of crushed trachea. These ex-
tracts will, in addition to extracellular fluid-phase proteins, 
contain a substantial amount of intracellular proteins. That this 
phenomenon is reflected in the pherograms is shown by Fig. 
2D. Whereas trachea fluid and eluate had a HPLC pattern 
similar to that of plasma, except for a lower content of large 
macromolecules, trachea lysate had a much higher fraction of 
low-molecular-mass macromolecules. This is to be expected 
because a large fraction of the latter consists of intracellular 
proteins. These experiments, together with the \(^{51}\)Cr-EDTA 
results, suggest that intracellular fluid without tracer accounted 
for the fluid that was added to the centrifugate discussed above.

During perturbations in tissue fluid balance, the composition 
of the interstitial fluid will change, and we could demonstrate 
that such changes occurring during overhydration and systemic 
inflammation were reflected in the isolated trachea interstitial 
fluid. Together, the tracer experiments, HPLC data, and 
induced interventions all suggest that samples representative 
for interstitial fluid can be isolated by centrifugation of trachea. 
The isolated fluid may also serve as a substrate for studies of 
biomarkers originating in the airways.

Comparison With Previous Studies

To our knowledge, there are no previous studies in which 
interstitial fluid was isolated from trachea in control conditions 
and in rats. Of major relevance, Nordin et al. (23) studied fluid 
balance in rabbit tracheal mucosa. To expose the microcircu-
lution and to immobilize the tissue, they made a longitudinal 
cut in trachea and clamped the tissue to the table. This proce-
dure generated an inflammation that was actually intended and 
resulted in a significant edema. Under these conditions, they 
were able to isolate lymph by direct puncture of terminal 
lymphatics and from protein concentration calculated an aver-
age COP in lymph of 19 mmHg, i.e., 91% of the plasma COP 
of 21 mmHg. Whether these values differed significantly is not 
known. They pointed out that a more gentle tissue handling 
resulted in a lower protein concentration in the isolated lymph, 
suggesting that the high protein concentration in lymph was the 
result of tissue manipulation. Although the experimental con-
ditions differed, our finding of an interstitial fluid COP in 
control situation 84% of that in plasma corresponds well to 
their data and may suggest that there is a high interstitial fluid 
protein concentration in control situation and that inflammation 
results in an increased filtration of water and protein (see 
Physiological Implications of the Study).

Although there are no other studies on trachea from euhy-
drated animals, Normandin et al. (24) were able to isolate fluid 
from the peribronchial-perivascular space in normally hydrated 
dogs using wick catheters, but the volume obtained in the 
control situation (<4 \(\mu\text{m}\)) was insufficient for analysis in their 
system. For the same tissue, Negrini et al. (22) were able to 
isolate fluid from the same space in euhydrated rabbits. They 
found a perivascular fluid protein concentration 58% of that in 
plasma, which was even lower in peribronchial fluid. The 
unexpected lower values may suggest that there are regional 
differences in interstitial fluid protein concentration and thus 
interstitial fluid COP in respiratory tissue. For lung tissue, 
lymph has been sampled and used as representative for inter-
stitial fluid (e.g., Refs. 26, 32). There may be fluid exchange in 
the lymph nodes; therefore, afferent lymph will be most re-
presentative for lung interstitial fluid (13, 20, 25). From these 
 studies, a protein concentration ratio between interstitial fluid 
and plasma of ~0.7 is found, i.e., somewhat lower than our 
corresponding ratio between COP in the two compartments of 
0.85. Still, these observations suggest that the interstitial fluid 
protein concentration is high in respiratory tissues compared 
with other tissues (for references, see Ref. 4).

BAL has been used extensively to monitor various processes 
occurring in respiratory tissue, mainly of inflammatory origin. 
BAL has its obvious advantages because of its versatility and
because samples are representative for more distal airways where the consequences of edema are more dramatic for airflow than in trachea. Here, we used this technique in LPS-induced inflammation to compare it with the centrifugation technique with respect to monitoring of inflammatory mediators. Because of the nature of the BAL technique, i.e., a large dilution volume, the concentration of inflammatory mediators was substantially higher in trachea centrifugate. Of note, the cytokine response pattern on LPS stimulation was, however, similar for the two techniques. This finding suggests that the response pattern is similar for upper and lower airways, again suggesting that signaling responses recorded in trachea interstitial fluid are representative for lower airways. There may be qualitative differences with respect to other proteins found in the two fluids, but this was not studied here. This question will be of importance for proteomic studies to identify mediators in such fluid, for example (34).

Physiological Implications of the Study

The findings of COP levels in trachea interstitial fluid that were 85% of that in plasma in control situation, corroborated by the protein concentration data, and 82% after an induced overhydration were somewhat unexpected. This is a significantly higher fraction than that of ~0.7 observed in lung lymph, as discussed above, as well as skin and muscle measured previously (42, 43) and gives information on fluid handling in this organ. A high interstitial fluid COP during control conditions may be a consequence of a very large capillary surface area in trachea (35, 36), allowing for a substantial diffusive transport of proteins. A possible low net capillary filtration will add to this effect (31). Whether such effects are sufficient to explain why an increased filtration induced by overhydration did not reduce lymph or interstitial fluid to plasma protein concentration ratio as observed in several other tissues (31) is not known. Although the filtration is increased, a large surface area will contribute to a significant dilution during overhydration also. Furthermore, the profuse lymphatic network (5) may contribute to a very effective removal of filtered fluid and proteins in this situation and to the maintenance of the relatively high interstitial fluid COP.

Interstitial fluid COP is one of the determinants of fluid filtration across the capillary wall. The importance, however, of interstitial fluid COP as measured here (“global” interstitial fluid COP) as a determinant of transcapillary fluid balance has been questioned because there may be significant protein gradients at the filtration barrier. This was initially suggested and shown for fenestrated capillaries (15, 17). Recently, Adamson et al. (1) verified that the same phenomenon also applies to nonfenestrated mesenteric capillaries in rats. These gradients are most pronounced during states of high filtration, whereas, at normal filtration, their model predicts that the effective COP is ~80% of that in the global interstitial fluid phase (i.e., interstitial fluid COP as measured in our study). Trachea has continuous or nonfenestrated capillaries, and the same phenomenon may apply here. Still, there is no doubt that this parameter is one of the predictors for the edema-preventive capacity of a tissue (4, 14). Previous studies by us in rat skin and muscle have shown that a decrease in interstitial fluid COP could counteract an increase in capillary pressure or a reduction in plasma COP of 8–10 mmHg (11, 29). Although we do not know at what interstitial fluid COP an edema will develop in trachea, the high interstitial fluid COP levels in control situations suggest that the interstitium is able to compensate for a significantly higher increase in net filtration pressure in trachea than in skin and muscle. Because avoidance of edema in the airways is of vital importance, the implication of this observation may be significant.

One significant finding of our study was the demonstration of a substantial local production of pro- and anti-inflammatory cytokines in trachea during a systemic inflammation induced by LPS. This effect was especially pronounced for IL-1β, which increased dramatically and was 16 times higher than the corresponding concentration in serum after LPS treatment, whereas IL-6 was twice that of serum in the same situation. A higher concentration in the tissue than in serum must mean that these cytokines are produced locally, an assumption that is supported by immunohistochemistry showing a significant increase in IL-1β- and IL-6-producing cells in the trachea interstitium. It is possible that the epithelium also contributes to local cytokine production because the airway epithelium has been shown to produce inflammatory cytokines on LPS stimulation (8, 28) and is also indicated by our immunohistochemistry data. Although the response to an inflammatory mediator may be complex and dependant on the species, the primary response to all of the main inflammatory mediators seems to be vasodilatation, resulting in thickening of the mucosa with edema and exudate as the final result (35, 36). Of note, the close association between cytokine-expressing cells and tracheal glands during LPS treatment suggests a stimulatory effect on mucous secretion that will add to swelling of the trachea.

Activated macrophages represent the major source of IL-1. These cells have a role in defense mechanisms following exogenous invasion of pathogenic stimuli and are most likely the origin for the observed IL-1β increase. They will also recruit monocytes and neutrophils when stimulated with LPS, enhancing the inflammatory reaction (19). TNF-α was higher in serum than in trachea fluid, in line with observations in skin (21). The liberated proinflammatory cytokines will induce local vascular leakage and edema (9, 18). LPS treatment, however, did not result in any edema in trachea in our experiments. A likely explanation for this observation is that LPS resulted in reduced capillary pressure and blood flow (6), and increased permeability alone is insufficient to cause edema unless capillary pressure is elevated (12).

The simultaneous increase in tissue fluid and serum IL-10 demonstrates the well-known stimulatory effect of LPS on the production of this anti-inflammatory cytokine (2). Furthermore, a higher level of IL-10 in serum than in trachea fluid suggests that this cytokine partly or fully originates from serum, a finding that is reasonable considering the preferential effect of LPS on IL-10 production in liver (2). Together, the present method for trachea fluid isolation may give significant new information regarding the process of edema generation during systemic inflammation caused by LPS.

In conclusion, we have shown that fluid can be isolated from trachea by centrifugation of excised tissue. We have validated that the isolated fluid is representative for trachea interstitial fluid providing that G forces ≥424 g are used. Our experiments show that the trachea interstitial fluid has a relatively high protein concentration and therefore high COP relative to plasma, again showing a significant potential for prevention of fluid accumulation and edema generation. During LPS-induced
systemic inflammation, there is a significant local production of proinflammatory mediators that may contribute to edema formation in trachea as well as lower airways. The present method thus represents a useful tool for studies of the microcirculation in the airways and may also be used in the search for airway specific biomarkers.

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