In Vivo Assessments of Mucus Dynamics in the Rat Lung Using a Gd-Cy5.5-Bilabeled Contrast Agent for Magnetic Resonance and Optical Imaging

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Dysfunctions in mucociliary clearance are associated with the accelerated loss of lung function in several respiratory diseases. Approaches enabling the in vivo visualization of mucus dynamics in rodents at high resolution and sensitivity would be beneficial for experimental lung research. We describe the synthesis and characterization of two bilabeled amino dextran-based probes binding specifically to mucin. Labeling of secreted mucus and of mucin in goblet cells in the lungs of lipopolysaccharide-challenged rats has been demonstrated in vivo with near-infrared fluorescence and MRI and confirmed by histology. The effects of uridine triphosphate were then studied in lipopolysaccharide-challenged rats by simultaneously administering the imaging probe and the compound. The data suggest that uridine triphosphate increased the mucociliary clearance, but at the same time induced a release of mucin from goblet cells, thus not contributing to the overall reduction of mucus in the lung. The approach outlined here enables one to derive information on mucus clearance, as well as secretion. Such a global view on mucus dynamics may prove invaluable when testing new pharmacological agents aimed at improving mucociliary clearance. Magn Reson Med 62:1164–1174, 2009. © 2009 Wiley-Liss, Inc.

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Mucociliary clearance is an important mechanism for removing inhaled particles, secretions, and cellular debris from the respiratory tract. A coordinated system of epithelial water and ion transport, mucin secretion, beating of cilia that line the respiratory tract, and cough, collectively termed “mucus clearance,” integrates to effectively remove inhaled particulates and thereby maintain lung sterility (1). Repeated lung infections and eventual respiratory insufficiency characteristic of human cystic fibrosis and primary ciliary dyskinesia illustrate the consequences of impaired mucus clearance. Altered mucus clearance likely contributes to the initiation, progression, and chronicity of other airway diseases characterized by inflammation and mucous secretory cell hyper/metaplasia that afflict millions worldwide, including chronic obstructive pulmonary disease (2, 3). A better understanding of the disease pathogenesis is required to identify specific therapeutic targets that, in turn, might lead to the rational design of drugs for treatment of obstructive pulmonary conditions (4, 5).

In humans and large animals, mucociliary clearance is most commonly assessed using inhaled radiolabeled aerosols and scintigraphy (6, 7). Although the assessment of mucociliary clearance by scintigraphy has been demonstrated in mice (8), terminal bronchoalveolar lavage fluid analyses remain the preferred method to derive information on mucus clearance in small rodents (9). The concentrations of fluorescent microspheres determined in lavage samples collected from animals sacrificed at different time points following the intratracheal (i.t.) administration of the microspheres allow one then to obtain a measure of particle clearance related to mucus clearance. For safety and ethical reasons, it is of great importance to avoid the use of radioactive materials and to reduce the number of terminal procedures in animal experimental research. In this context, it would be of interest to have a noninvasive method to obtain, in small rodents, information on the mucus dynamics at high resolution without employing radioactivity. Moreover, if the technique can be translated into the clinic, it may improve the ability to diagnose and stage disease and to assess treatment efficacy.

Animal models have been established in an attempt to mimic and study specific aspects of human respiratory disease (10, 11). For instance, administration of the endotoxin, lipopolysaccharide (LPS), a bacterial macromolecular cell surface antigen, to animals induces in the lung an inflammatory response that replicates many of the important features of human chronic obstructive pulmonary disease, such as pulmonary neutrophilia, goblet cell hyperplasia in the airway epithelium, and the secretion of airway mucus (12), a viscous colloid containing antiseptic enzymes (such as lysozymes) and immunoglobulins.

MRI can detect a mucus hypersecretory phenotype in rats after LPS challenge (13, 14). For Brown Norway (BN) rats, it has been shown that sustained signals were apparent in the lungs until day 16 after the i.t. instillation of...
LPS. The signal had two components: one, of diffuse appearance and higher intensity, was particularly prominent up to 48 h after LPS; the second, showing an irregular appearance and weaker intensity, was predominant later. Bronchoalveolar lavage fluid analysis indicated that generalized granulocytic (especially neutrophilic) inflammation was a major contributor to the signal at the early time points, with mucus being a main factor contributing at the later time points (13-15).

In the present work we show the feasibility of assessing in vivo the mucus dynamics in the lungs of LPS-challenged BN rats with MRI and near-infrared fluorescence (NIRF) imaging by administering a Gd- and Cy5.5-labeled amino dextran aimed at binding specifically to mucus. The rationale for the probe synthesis was based on the fact that dextran is a polysaccharide that competes with mucous glycoproteins for hydrogen binding sites (16). Details of the probe synthesis are provided. Following its chemical analysis, the probe was characterized ex vivo in mucus samples from an asthmatic individual and in vivo in LPS-challenged BN rats. In vivo characterization comprised the use of MRI and NIRF optical imaging followed by histologic validation. Although the majority of the in vivo studies were performed with MRI rather than NIRF, the double label enabled the histologic characterization using confocal microscopy. Finally, the probe was adopted for studying in vivo with MRI the effects of uridine triphosphate (UTP) in LPS-challenged rats. UTP is known to increase the rate of mucus clearance in healthy humans, as well as in patients suffering from chronic bronchitis or cystic fibrosis (17,18).

**MATERIALS AND METHODS**

Experiments were carried out with the approval of the Veterinary Authority of the City of Basel (license number 1989).

**Study Rationale**

The objective of the present study was to show the feasibility of improving the detection of mucus by MRI in the lungs of LPS-challenged rats with a contrast agent in powder or in liquid form. I.t. administration of a powder or a liquid usually led to completely different distribution patterns of the material in the lung, as illustrated in Fig. 1. These ex vivo NIRF images were obtained 10 min after i.t. dosing of Cy5.5-labeled lactose administered in liquid form or as a powder. It can be clearly seen that while the powder was predominantly localized in the main airways, the liquid distributed more evenly, reaching also distal airways. Therefore, instead of comparing the effectiveness of the two imaging agents, we limited our attention to the ability of both probes to label mucus. In a first step, in order to avoid signals from the vehicle, a probe in powder form (BCR250) has been tested. In a second step, an agent (BCR249) has been developed for administration in liquid form, together with a compound (UTP) modulating the clearance of mucus. This approach ensured the same distribution of the therapeutic agent (UTP) and the imaging agent (BCR249) in the lung. The protocol to induce a mucus hypersecretory phenotype in the airways of BN rats has been reported earlier (13,14).

**Animals**

Male BN rats (n = 54), weighing approximately 200 g, were supplied by IFFA CREDO (L’Arbresle, France). Upon arrival, animals were kept at an ambient temperature of 22 ± 2°C under a 12-h normal-phase light-dark cycle and fed NAFAG® pellets (Nahr- und Futtermittel AG, Gossau, Switzerland) for a week before starting the experiments. Drinking water was freely available.

Animals were divided into following studies: (i) NIRF studies of powder versus liquid distribution in the lungs (n = 2 rats). Measurements performed 10 min after material administration. (ii) Effects of BCR250 (n = 8 rats). Two naïve rats were examined by MRI 1 h after BCR250 administration. Six animals received BCR250 24 h after LPS challenge. They were examined by MRI at baseline (1-2 days before LPS) and at time points −20 min, 1 h, 12 h, 24 h, and 48 h with respect to BCR250 administration. (iii) Effects of BCR249 (n = 16 rats). Two naïve rats were examined by MRI 24 h before (baseline) and 1 h after BCR249 administration. Fourteen animals were challenged with LPS and received either BCR249 (n = 7) or vehicle (saline, n = 7) 24 h later. They were examined by MRI at baseline (1-2 days before LPS) and at time points −20 min, 1 h, 4 h, 7 h, 24 h, and 48 h with respect to BCR249 (or vehicle) administration. Four rats were additionally examined by NIRF at 24 h after BCR249. (iv)
Effects of UTP and BCR249 (n = 28 rats). All 28 animals were challenged by LPS and received 24 h later UTP together with BCR249 (or the vehicle). Animals were examined by MRI at baseline (1-2 days before LPS) and at time points 2 h, 1 h, 4 h, 7 h, and 24 h after UTP and probe administration.

LPS Challenge

In order to induce mucus hypersecretory phenotype in the airways (13,14), LPS from *Salmonella typhosa* (Sigma, Dorset, UK; dissolved in saline to a concentration of 1 mg/kg; solution volume 0.2 mL) was administered i.t. to the rats under gas anesthesia (3% isoflurane; Abbott, Cham, Switzerland) and the animals were allowed to recover.

Contrast Agents

*Materials and Characterization*

The commercial materials 1H-benz[e]indolium, 2-[5-[3-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-1,3-dihydro-1,1-dimethyl-6,8-disulfo-2H-benz[e]indol-2-ylidene]-1,3-pentadien-1-yl]-3-ethyl-1,1-dimethyl-6,8-disulfo—inner salt, sodium salt (1:3) (Cy5.5 NHS ester; GE Healthcare, Otelfingen, Switzerland), 2-(p-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-N,N,N',N"-tetraacetic acid (p-SCN-Bz-DOTA; Macrocyclics Inc., Dallas, TX), amino dextran (Molecular Probes, Invitrogen, Basel, Switzerland), and gadolinium chloride hexahydrate (Sigma-Aldrich, Buchs, Switzerland) were used as received. Measurements of ultraviolet-visible spectrophotometry spectra and determination of Cy5.5 concentrations were performed with a Lambda 25 spectrophotometer (Perkin-Elmer Life and Analytical Sciences, Inc., Waltham, MA). Absorption spectra were measured in the range 200-850 nm, with a slit width of 2 nm and a scan rate of 120 nm/min, using phosphate-buffered saline in the reference channel. Gadolinium concentration was assessed at Solvias AG (Basel, Switzerland) by inductively coupled plasma optical emission spectrometry using the ICP-optical emission spectrometer Optima 3000 DV (Perkin-Elmer Life and Analytical Sciences, Inc. Waltham, MA). Proton NMR spectra were recorded at 400 MHz on a Bruker BioSpin (Bruker Biospin AG, Fällanden, Switzerland) spectrometer using deuterium oxide as solvent and chemical shift reference.

*Synthesis*

A schematic representation of the chemical synthesis of the probes is provided in Fig. 2.

**BCR250.** Amino dextran10 (425 mg, 0.042 mmol; molecular weight: 10 kDa) was dissolved over a period of 2 h (swelling) in NaHCO₃ (0.1 M, 15 mL) and adjusted to pH 9 by Na₂CO₃ (0.1 M). After adding Cy5.5 NHS ester (1.1 eq., 46.9 mg, 0.046 mmol) dissolved in NaHCO₃ (0.1 M, 0.5 mL), the resulting mixture was stirred for 15 min at room temperature. pSCN-Bz-DOTA (6 eq., 177 mg, 0.253 mmol) was dissolved in NaHCO₃ (0.1 M, 5 mL) adjusted to pH 9 by Na₂CO₃ (0.1 M) and added to the reaction mixture that was stirred an additional 18 h at room temperature. Part of the reaction mixture (0.2 mL) was purified by size exclusion chromatography (Sephadex, bidest water). After lyophilization the labeling ratio of the DOTA-ligand was estimated by proton NMR using specific signal integrals for the DOTA ligand (4 Ar-H) and the dextran subunits (e.g., O-C≡O—O) (Fig. 3a). The remaining solution was purified by ultrafiltration (molecular-weight cutoff 5,000, PES, 5 × NaHCO₃, 0.1 M) until the filtrate appeared colorless. The buffer was exchanged (2 × citrate buffer, 0.1 M, pH 8), and after dissolving gadolinium chloride hexahydrate (5 eq., 78.4 mg, 0.211 mmol) in citrate buffer (0.1 M) and adjusting to pH 9 by NaOH (1 M) the reaction mixture was stirred for 72 h at room temperature. Purification was carried out by ultrafiltration (molecular-weight cutoff...
5.000, polyethersulfone (PES), 5× citrate buffer, 0.1 M; pH 8, 5× bidest water) until the filtrate was free of unbound gadolinium ions (xylene orange test at pH 6). BCR250 was obtained as blue powder after lyophilization; elemental analysis (inductively coupled plasma optical emission spectrometry): 4.49% Gd (approximately 4 Gd per molecule). A Cy5.5/dextran ratio of 0.75 was determined by an ultraviolet concentration assay at absorbance maximum (λ_max = 678 nm) of Cy5.5 (Fig. 3b) using a stock solution at a defined conjugate concentration.

BCR249 was synthesized as described above for BCR250 starting from amino dextran 70 (molecular weight: 70 kDa) using 36 eq. pSCN-Bz-DOTA and 30 eq. gadolinium chloride hexahydrate; elemental analysis (inductively coupled plasma optical emission spectrometry): 4.42% Gd (approximately 24 Gd per molecule). Cy5.5/dextran ratio (λ_max = 678 nm) = 0.55. BCR249 was dissolved in physiologic saline (20 mg/mL) when synchronously administered with UTP or its vehicle.

Milling of the powder form of BCR249 led to particles not small enough to be used with a syringe jet. On the other hand, milling of the powder form of BCR250 led to particles smaller than 5 μm, which could be administered with a syringe jet.

### Drug Treatment

In order to guarantee the same distribution for both the compound and the contrast agent in the rat lung, the imaging probe was administered at the same time as UTP. Thus, UTP (60 mM; Sigma-Aldrich, Buchs, Switzerland) was dissolved in physiologic saline (vehicle) with or without added BCR249. A curved cannula built in house to allow spraying solutions was mounted on a 1-mL syringe. At 24 h after LPS challenge, aliquots (0.2 mL) of the solutions were sprayed i.t. for 2-3 sec.

### Experimental Design

In the first set of experiments, four groups of four rats each were designed to determine the most appropriate form of the contrast agent, as a powder or a solution form, in the context of our study. In this regard, rats were challenged with LPS (1 mg/kg i.t.), 24 h before receiving BCR249 (4 mg dissolved in 0.2 mL of physiologic saline), BCR250 (4 mg) or their respective controls: 0.2 mL of saline and 4 mg of lactose (Sigma-Aldrich, Buchs, Switzerland). All compounds or vehicles were administered i.t. The 16 rats were examined by MRI.
A second set of four experiments was designed with the aim of characterizing in rats challenged with LPS (1 mg/kg i.t.) the effects of UTP on contrast agent-enhanced MRI signals. In order to ensure the same spatial distribution for the therapeutic and the contrast agent in the lung, both were combined. Upon i.t. administration of UTP and the probe 24 h after LPS challenge, the global fluid signals changes detected by MRI were governed by three factors: (i) effects of UTP; (ii) effects of the UTP-vehicle; and (iii) effects of the contrast agent. In order to separate the effects of UTP on labeled mucus, two experiments were performed in separate groups of LPS-challenged animals: experiment I: UTP (60 mM) + BCR249 (4 mg) in physiologic saline (0.2 mL) administered i.t. 24 h after LPS (n = 7 rats); experiment II: UTP (60 mM) in physiologic saline (0.2 mL) administered i.t. 24 h after LPS (n = 7 rats).

The volumes of MRI fluid signals in the lungs from experiment II were then subtracted on a time point by time point basis from those corresponding to experiment I. The subtracted data then reflected the effect of UTP on contrast agent–labeled mucus. The same procedure was repeated for vehicle treatment in two additional groups of LPS-challenged rats: experiment III: BCR249 (4 mg) in physiologic saline (0.2 mL) administered i.t. 24 h after LPS (n = 7 rats); experiment IV: physiologic saline (0.2 mL) administered i.t. 24 h after LPS (n = 7 rats).

The volumes of MRI fluid signals from experiment IV were then subtracted on a time point by time point basis from those corresponding to experiment III. The subtracted data then reflected the effect of saline on the dynamics of contrast agent–labeled mucus.

Further experiments were performed on two naïve rats to verify whether i.t. administration of BCR249 (4 mg) in physiologic saline (0.2 mL) might result in any adverse effects.

MRI
With the exception of two animals used to show the difference between i.t. dosing of powder and fluid (Fig. 1), rats (n = 44) have been examined by MRI. Measurements were carried out with a Biospec 47/40 spectrometer (Bruker Medical Systems) operating at 4.7 T. A gradient-echo sequence with repetition time 5.6 ms, echo time 2.7 ms, band width 100 kHz, flip angle of the excitation pulse approximately 15°, field of view 6 × 6 cm², matrix size 256 × 128, and slice thickness 1.5 mm was used throughout the study. Neither cardiac nor respiratory gating was applied for data acquisition. A single-slice image was obtained by computing the two-dimensional Fourier transform of the averaged signal from 60 individual image acquisitions and interpolating the data set to 256 × 256 pixels. There was an interval of 530 ms between individual image acquisitions, resulting in a total acquisition time of 75 sec for a single slice. A birdcage resonator of 7-cm diameter was used for excitation and detection. During MRI measurements, rats were placed in supine position and anesthetized with 2% isoflurane in a mixture of O₂/N₂O [1:2], administered via a face mask) and then ex vivo after the rat had received an overdose of pentobarbital (250 mg/kg i.p.) and lungs had been excised. For the in vivo acquisitions, rats were in supine position. Three laser diodes operating at 660 nm, with a power of 10 mW/cm², were used for fluorescence excitation. The fluorescent light emitted from the lungs was detected by a charge-coupled device camera (Hamamatsu Photonics, Schüpfen, Switzerland) equipped with a focusing lens system (macro lens 60 mm, 1:2.8; Nikon AG, Egg, Switzerland). The charge-coupled device featured low noise and low dark signal, enabling low-light-level detection, as well as long integration times. The matrix size of the images was 532 × 256 pixels. A hard cutoff filter (700-nm long-pass) was used for fluorescence detection. Data acquisition (i.e., integration) times ranged from 0.5 to 2.0 sec, depending on the intensity of fluorescence. The experiment was controlled using the SYNGO® software (Siemens Medical Solutions, Erlangen, Germany).

Histology
Immediately following an MRI session, rats were sacrificed by an overdose of pentobarbital (250 mg/kg i.p.). Lungs were removed from the thorax and then immersed in 10% neutral formalin buffer for 72 h. After fixation, transverse sections were cut through the median part of the left, the right apical, the median, and the caudal lobes. Sections were dehydrated through increasing graded ethylic alcohol and processed into paraffin wax overnight. Three serial slices of 3-μm thickness were cut from each section. The following stains were then applied: (i) hematoxylin/eosin to assess general morphology; and (ii) periodic acid–Schiff/Alcian blue reaction to detect mucus and goblet cells (20). Slices were examined with a light microscope (DMR BE; Leica Microsystems, Gatterbrugg, Switzerland) connected to a video camera (Progres/3008; Jenoptik LOS, Eching, Germany). One slice was dewaxed in xylene, rehydrated though a series of decreasing graded ethylic alcohol to distilled water. The nuclei were counterstained.
with propidium iodide and section mounted with Gel Mount aqueous mounting medium (Biomeda, Foster City, CA). The section was placed under the objective of a confocal microscope with spectroscopic detection (TCS SP2; Leica Microsystems, Glattsbrugg, Switzerland). For the detection of Cy5.5, an appropriate laser (excitation 633 nm) and filter block were used. Image acquisition time was 9 sec.

Sputum Collection

For ex vivo analyses, sputum has been collected from an asthmatic individual over the period of 1 week and stored at 2°C in five different test tubes. Imaging was performed 1 h after addition of vehicle or contrast material to the vials. The acquisition parameters were the same as those adopted for the in vivo acquisitions.

Statistics

MRI data were analyzed by SYSTAT 12 (Systat Software, Inc., San Jose, CA). We used an extension of analysis of variance (ANOVA), called "mixed model analysis" or "ANOVA with random effects", to take into account the longitudinal structure of the data. For multiple comparisons, a Bonferroni correction followed the ANOVA.

RESULTS

Characterization of the Imaging Probes

Elemental analysis of BCR249 showed 4.42% Gd (24 × Gd per molecule). The Cy5.5 ratio determined by an ultraviolet-absorbance concentration assay at wavelength maximum of the dye was 0.55 Cy5.5 per molecule. The ability of the agent to label mucus is illustrated in Fig. 4, showing that a selective increase of mucus signal with respect to the background was observed by both MRI and NIRF when BCR249 was added to a vial containing sputum obtained from an asthmatic individual. No selective enhancement of mucus has been observed by either MRI or NIRF when other agents were added to test tubes.

For BCR250, on the other hand, elemental analysis showed labeling of 4.49% Gd (4 × Gd per molecule), and ultraviolet analysis determined a Cy5.5 ratio of 0.75 × Cy5.5 per molecule. As i.t. instillation of solutions leads temporarily to MRI signal enhancement in the rat lung, it was decided to prepare the contrast agent as a dry powder as well. Milling of BCR250 led to particles that were small enough (90% under 5 μm) (Fig. 5) to be applied i.t. by a 5-ml-syringe jet through a 1.6-mm-diameter cannula.

Effects of the Imaging Agents In Vivo

In a first series of experiments, the imaging probe in powder form (BCR250) was tested in endotoxin-exposed rats. Fig. 6a shows representative MRI transverse sections through the thoracic region of a rat at various times after i.t. exposure to LPS (1 mg/kg). At 24 h following LPS, MRI detected discrete signals of weak intensity in the lungs, shown to be related to secreted mucus in previous publications (13,14). I.t. administration of BCR250 at this time point (and immediately after the imaging session) resulted in a significant increase of MRI signals until 24 h later (Fig. 6b). Histology from the anatomic region displaying high

![FIG. 4. Ex vivo effects of BCR249. Magnetic resonance (left panel) and NIRF (right panel) images of vials containing sputum from an asthmatic volunteer, with addition of 0.2 mL of physiologic saline, Gd-DOTA, Cy5.5, Cy5.5-labeled cysteine (Cy5.5-Cys), or BCR249 (20 mg/mL). MRI and NIRF revealed a selective signal increase of mucus contained in the sputum only when BCR249 was added. Parameters of the MRI acquisition were the same as for the images obtained in vivo. Imaging was performed 1 h after addition of the agents to the vials.](image1)

![FIG. 5. Electron microscopy of milled BCR250. Most particles were smaller than 5 μm.](image2)
intensity MRI signals, performed at 1 h after BCR250 administration, demonstrated that the agent bound to mucin that had been secreted (Fig. 6c). The signal from labeled secreted mucus detected by confocal microscopy had a diffuse nature; however, the technique also revealed compact signals presenting the form and the extent of goblet cells (Fig. 6d), suggesting that to some degree mucin present in these cells has been labeled as well. No mucus was labeled when the vehicle for BCR250 was administered (Fig. 6e). At 1 h after i.t. administration of BCR250, no particular signals were detected in the lungs of naïve BN rats (Fig. 6f).

Under in vivo conditions, an enhancement of MRI signals elicited in the lungs by endotoxin provocation was also ob-
served when the probe in solution form (BCR249) was given 24 h post-LPS and still detected 24 h later (Fig. 7a). At this time point, NIRF detected in vivo a prominent signal in the same region where enhancements had been detected by MRI, an observation that was confirmed by optical imaging of the isolated lungs (Fig. 7b). Fig. 7c illustrates the course of MRI signals detected in the lungs of LPS-challenged rats that received BCR249 or its vehicle (saline) at 24 h after endotoxin exposure. I.t. administration of the agent led to a prominent increase in signal volume in the first hour following its delivery, from 0.33 ± 0.05 mL, in response to endotoxin to 0.64 ± 0.09 mL after the contrast agent administration. Six hours later, the signal volume had decreased to 0.38 ± 0.07 mL, close to precontrast agent administration levels, probably because of mucus clearance. Nonetheless, the signal volumes were still significantly augmented 48 h later (0.28 ± 0.06 mL) when compared to those signals observed after vehicle (saline) instillation (0.04 ± 0.03 mL), as shown in Fig. 7c. At 1 h after i.t. administration of BCR249, no particular signals were detected in the lungs of naive BN rats (Fig. 7d).
Effects of the Nucleotide UTP on LPS-Induced Mucus Hypersecretion

The next experiment aimed at studying the fluid dynamics in the rat lung when UTP, known to increase mucus clearance, was administered i.t. 24 h after LPS. In order to ensure that UTP and the imaging probe had the same distribution in the lungs, both agents were administered simultaneously. Fig. 8a summarizes the course of signals detected by MRI in the lungs of naive BN rats following the administration of UTP or its vehicle at 24 h after endotoxin. As shown in Fig. 8a, within the first hour following its administration, and when compared to its vehicle, an increase of 0.21 mL of the global fluid volume was detected in the lung of UTP-treated rats. The effect of UTP on mucus clearance is better illustrated by the dynamics of mucus labeled by the contrast agent (Fig. 8c), obtained by subtracting the curves of Fig. 8a data that were collected in the same conditions in another group of LPS-challenged rats but without contrast agent addition (Fig. 8b). Because the probe had been demonstrated earlier to bind specifically to mucus, it can be assumed that the subtraction of fluid signals obtained without the probe from signals detected in the presence of the agent provides a means to assess the dynamics of mucus labeled by the contrast agent. Thus, the subtracted data show that UTP had an early effect (significant at 4 h after its administration) on the clearance of labeled mucus, whereas the vehicle-treated animals demonstrated maintained probe-specific signals until 24 h (Fig. 8b). Histologic analysis confirmed that UTP administration led to emptying of goblet cells and to an accelerated clearance of labeled mucus from the airways lumen (Fig. 8d).

DISCUSSION

The present study shows that, in the lungs of LPS-treated rats, amino dextran–based imaging agents bound specifically to mucus. Careful histologic examination revealed that secreted mucus, and to a certain extent, also mucin in goblet cells, was labeled by the contrast agents. This suggests that the time course of MRI signals in the lungs of LPS-challenged animals following administration of the contrast agents not only reflected the mechanics of mucociliary clearance but also the resolution of mucus plugging. Furthermore, as the agents also labeled mucin in goblet cells, it is conceivable that the continuous secretion of mucus following LPS contributed as well to sustain the MRI signal.
The volumes of MRI hyperintense signals in the lungs of LPS-challenged rats following BCR249 or BCR250 administration were larger than those previous to contrast agent administration (Figs. 5 and 6). Several reasons might have contributed to this observation: (i) increased sensitivity for mucus detection provided by the agents; (ii) mucus secretion is a dynamic process. Thus, it cannot be excluded that there was mucus release between the time point of administration of the contrast agent and the next MRI acquisition. The released mucus could have contributed to an enlargement of the area of hyperintense signal; and (iii) small molecular weight dextran (< 5000 Da) is used as mucolytic agent (16,21). Although we used dextran of higher molecular weight, it is possible that the agent itself caused some mucolysis, leading to an enlargement of the volume, in addition to an increase in signal intensity of mucus-related signals.

The clearance of particles from the airways is a complex process. Upon inhalation, particles deposited on airways surfaces are cleared by various mechanisms. Depending on their initial site of deposition and size, a fast- and a slow-clearance phase has been observed in particle retention experiments. The fast-clearance phase has traditionally been interpreted as tracheobronchial, whereas the slow-clearance phase is commonly attributed to mechanical clearance from the alveolar region by alveolar macrophages or by transcytosis through the alveolar epithelium and subsequent transport to the bloodstream and associated lymph nodes (22,23). While there is general agreement that mucociliary transport is the principal clearance mechanism in the tracheobronchial region during the first 24 h after exposure (24), experiments in rats and in humans suggested that the slow-clearance phase may be partly explained by a delayed clearance from peripheral bronchiolar airways in an asymmetric lung structure (25,26). Moreover, mucus discontinuities mainly found in intermediate and distal airways of the tracheobronchial compartment, mucus production in proximal and bronchial airways, an eventual delay of the mucociliary transport at carinal ridges of airway bifurcations (26), and rheological properties of mucus (27) are factors that also contribute to its clearance. In all generated clearance velocity models, mucociliary clearance is completely terminated within 24 h after exposure, consistent with the experimental evidence. Implementation of a slow bronchial clearance phase predicts a long-term retention fraction, which is fully cleared from the lung after several weeks (26,27).

Tracheal mucociliary clearance velocities in rats presented in the literature display a wide range of values, presumably because of differences in the techniques and the anesthesia used for measurements. Values range between 1.9 and 8.1 mm/min (26,28,29). A comparison of these values with the rates of MRI signal resolution presented here suggests that what we detected with MRI were events primarily related to the slower, delayed clearance phase.

Exogenous nucleotides have been shown to modulate several physiologic activities that are vital to the mucociliary apparatus. Adenosine triphosphate and UTP induce chloride secretion and water movement into the airway surface liquid, which hydrates mucus and optimizes periciliary fluid viscosity (16,30,31), both crucial mechanisms for an efficient ciliary beating (24,32). Other studies demonstrated direct stimulatory effects of UTP on ciliary beat frequency (30,33), as well as increased submucosal gland secretion (30) and mucin emptying from goblet cells (34-36) induced by UTP.

In agreement with the literature (17,18), for the UTP treatment study, our data suggest an increase of mucociliary clearance induced by the nucleotide since the calculated volume of signal of labeled mucus was lower for UTP-treated rats than for the vehicle group (Fig. 8c). These observations are supported by histology at 24 h after UTP, demonstrating the presence of contrast agent–labeled mucus in lung sections from vehicle-treated animals only (Fig. 8d). However, a distinct MRI signal increase was observed within 1 h post-UTP, higher than that following vehicle administration (Fig. 8a,b). Based on previous results showing that MRI noninvasively detects LPS-induced mucus hypersecretion (13,14), we hypothesize that the signal increase observed after UTP was due to release of mucus induced by the nucleotide. Histology sustains this view as goblet cells were empty of mucin 24 h post-UTP (Fig. 8d). These results agree with the view that increased mucus secretion is often coupled with a higher mucus clearance (37), which may be a protective response of the lung and to the fact that secretion of mucins from airway goblet cells appears to be regulated primarily by the agonists adenosine triphosphate and UTP, which activate P2Y2 purinoceptors and phospholipase C to initiate inositol triphosphate (IP3)/Ca2+ and diacylglycerol/protein kinase C intracellular signaling cascades (31,38,39). Overall, despite showing an increased clearance of labeled mucus by UTP, our data question the benefit of the nucleotide in the model. Indeed, the MRI signal course displayed in Fig. 7a shows that the overall fluid content 24 h after UTP and later was the same for UTP- and vehicle-treated rats, suggesting no beneficial effect of the compound at the dose used. It remains to be verified whether doses of UTP could be found for which the balance would be shifted away from mucin secretion from goblet cells toward clearance of mucus. The outlined imaging approach would be very useful to guide such studies.

In summary, the results presented here indicate the advantage of using MRI and a probe for specifically labeling mucus: the technique provides at the same time a global view on overall fluid dynamics in the lungs, as well as specific information on the mucus dynamics. Optical imaging in combination with the mucus probe has potential to provide information on mucus clearance. Both types of information may be fundamental in gaining insights into the therapeutic value of compounds aimed at influencing the dynamics of mucus in the lung. Although the probe has been primarily conceived for use in experimental research in small rodents, imaging of the mucus dynamics as discussed here entails attractive possibilities for clinical translatability. While MRI has already been recognized as an important diagnostic tool of lung diseases in the clinics (40), fiberoptic bronchoscopy is a promising approach for optical molecular imaging. Fiberoptic catheters are applicable to humans, are handheld, and do not require large imaging equipment. They are capable of visualization deep in fourth-order bronchi. The combination of both techniques should also be feasible. Future work is neces-
sary to verify whether the approach outlined here is translatable to the clinics and if it is able to address key biologic issues in lung pathophysiology.

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