

Near-Infrared Fluorescence Imaging and Histology Confirm Anomalous Edematous Signal Distribution Detected in the Rat Lung by MRI After Allergen Challenge

Bruno Tigani, PhD,¹ Hans-Ulrich Gremlich, PhD,¹ Catherine Cannet, MSc,¹ Stefan Zurbrugg, BSc,¹ Harry Karmouty Quintana, BSc,^{1,2} and Nicolau Beckmann, PhD^{1*}

Purpose: To address the issue concerning the predominant location, on the left anatomic side, of edematous signals detected by magnetic resonance imaging (MRI) in the lungs of actively sensitized rats following intratracheal (IT) allergen challenge.

Materials and Methods: Near-infrared fluorescence (NIRF) imaging was used to detect the lobular distribution in the lungs of normal rats of an IT instilled fluorescent dye, Cy5.5. Actively sensitized Brown Norway rats were examined by MRI 24 hours after IT administration of ovalbumin. The perivascular edema was quantified by histology in the different lobes of lungs removed from the same animals immediately after the MRI acquisitions.

Results: An uneven distribution of Cy5.5 was found, predominantly on the left lobe, paralleling the localized development of allergic pulmonary inflammation in the left lobe detected as edematous signal by MRI and confirmed by histology. The patterns of the distributions of the dye between and within the lobes were very similar to those of perivascular edema assessed histologically.

Conclusion: The data indicate a relationship between the molecular deposition of the dye detected by NIRF in the lungs and the distribution of allergen eliciting the development of pulmonary inflammation in actively rats. The combination of MRI with NIRF imaging may provide important information in preclinical pharmacologic research in the area of airway diseases. While MRI is able to address the effects of compounds on the inflammatory response in models of airways diseases, NIRF imaging may provide

important insights on drug distribution and interaction in the lung, being thus suited for molecular imaging studies.

Key Words: asthma; lung; magnetic resonance imaging (MRI); molecular imaging; near-infrared fluorescence (NIRF) imaging

J. Magn. Reson. Imaging 2004;20:967–974.

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THE NONINVASIVE detection by magnetic resonance imaging (MRI) of an edematous signal associated with allergic inflammation induced in the lungs of actively sensitized Brown Norway (BN) rats challenged with ovalbumin (OVA) has been recently described (1). This signal has been shown to significantly correlate with the inflammatory parameters in the bronchoalveolar lavage fluid (2), and with the perivascular edema quantified by histology (3), representing a key noninvasive indicator of allergic inflammation. Because edema formation is an integral component of pulmonary inflammation, MRI provides a noninvasive means of monitoring the course of the inflammatory response in animal models of lung inflammation in rats, and should prove invaluable in profiling antiinflammatory drugs in vivo (3–5). One particularly interesting aspect of the signal detected by MRI is its predominant location on the left side of the animals despite the fact that allergen was administered intratracheally (IT), before the bifurcation of the trachea.

Over the last decades, several investigators have attempted to evaluate in animals the pulmonary distribution patterns of particles administered via different routes of delivery (6–9). Histologic analysis demonstrated that IT instillation and aerosol inhalation of particles led to a lobar difference in their deposition. Of note is that the distribution was considerably more nonuniform when the particles were instilled.

The emergence of imaging techniques is bringing new perspectives to the profiling of drug candidates (10). In particular, imaging measurements of biodistribution and kinetic may provide important information for the drug development process, such as the minimum nec-

¹Novartis Institutes for BioMedical Research, Discovery Technologies, Basel, Switzerland.

²Sackler Institute of Pulmonary Pharmacology, King's College London, London, UK.

Contract grant sponsor: 3R Research Foundation, Muensingen, Switzerland; Contract grant number: 82-02.

*Address reprint requests to: N.B., Novartis Institutes for BioMedical Research, Discovery Technologies, Lichstr. 35, WSJ-386.2.09, CH-4002 Basel, Switzerland.

E-mail: nicolau.beckmann@pharma.novartis.com

Received June 3, 2004; Accepted August 4, 2004.

DOI 10.1002/jmri.20203

Published online in Wiley InterScience (www.interscience.wiley.com).

essary drug deposition for inducing an effect, the identification of the region in which deposition is important, or its duration of action (11).

The present article reports the results of comparing the perivascular edema quantified by histology in the different lobes of lungs removed from rats 24 hours after OVA challenge and the pattern of lung lobular distribution of IT instilled particles of a dye assessed by near-infrared fluorescence (NIRF) imaging measurements. The aim was to address the question of a possible relationship between the molecular deposition of the dye and the distribution of allergen eliciting the development of pulmonary inflammation.

MATERIALS AND METHODS

All experiments were carried out according to the Swiss federal regulations for animal protection.

Animals

Male BN rats (Iffa-Credo, L'Arbresle, France) weighing about 280 g were used throughout the study. They were housed in a temperature- and humidity-controlled environment, having free access to standard rat chow and tap water.

Sensitization

OVA (20 µg/mL; Fluka, Buchs, Switzerland) was mixed (30 minutes on ice) in a blender (Polytron Kinematica; Brinkmann Instruments Inc., Westbury, NY) with aluminum hydroxide (20 mg/mL) and administered subcutaneously (0.5 mL per animal). The sensitization procedure was repeated on days 15 and 22. Sensitized animals were used between days 29 and 35.

Experimental Protocols

Actively sensitized BN rats were divided in two groups. In a first group, animals ($N = 8$) were anesthetized with 4% Forene (Abbott, Cham, Switzerland). OVA (0.3 mg/kg dissolved in saline, 0.2 mL per animal) was administered IT and the animals allowed to recover. Animals were imaged by MRI at 24 hours after OVA, and killed immediately after with an overdose of pentobarbital (250 mg/kg intraperitoneally) for histologic analyses.

Rats ($N = 8$) from the second group were anesthetized with 4% Forene for IT administration of 0.2 mL of a 0.001% solution of the fluorescent dye Cy5.5 (Amersham Biosciences, Freiburg, Germany) in saline. Following administration of the dye, animals were allowed to recover from anesthesia. They were killed approximately an hour later with an overdose of pentobarbital (250 mg/kg intraperitoneally). Lungs were then fixed by slow in situ inflation of approximately 5 mL of 10% phosphate-buffered neutral formalin, pH 7.2, administered via a trachea cannula. Following removal of the lungs, fluorescence reflectance images were recorded using the bonSAI Siemens small animal imager prototype (Siemens Medical Solutions, Erlangen, Germany).

MRI

Measurements were carried out in vivo with a Biospec 47/40 spectrometer (Bruker Medical Systems, Karlsruhe, Germany) operating at 4.7 T, equipped with an actively shielded gradient system. The operational software of the scanner was Paravision® (Bruker Medical Systems). A gradient-echo sequence with repetition time (TR) 5.6 msec, echo time (TE) 2.7 msec, bandwidth 100 kHz, flip angle of the excitation pulse approximately 15°, field of view (FOV) 6×6 cm², matrix size 256×128 , and slice thickness 1.5 mm was used throughout the study. A single slice image was obtained by computing the two-dimensional Fourier transform (2DFT) of the averaged signal from 60 individual image acquisitions and interpolating the data set to 256×256 pixels. There was an interval of 530 msec between individual image acquisitions, resulting in a total acquisition time of 75 seconds for a single slice. The entire lung was covered by 16 consecutive slices and the total examination time per rat was about 25 minutes (including positioning of the animal). A birdcage resonator of 7 cm diameter was used for excitation and detection. During MRI measurements, rats were anesthetized with 2% Forene in a mixture of O₂/N₂O (1:2), administered via a facemask. During image acquisition, animals breathed spontaneously, and neither respiratory nor cardiac gating was applied. The body temperature of the animals was maintained at $37 \pm 0.5^\circ\text{C}$.

MR Image Analysis

The volume of the edematous signals was determined using a semiautomatic segmentation procedure described in detail previously (1) applied to the 16 slices measured per rat. Segmentation parameters were the same for all analyzed images, chosen to discriminate regions corresponding to high intensity signals. Because the signals from edema and vessels were of comparable intensities, the volume corresponding to the vessels was assessed on baseline images and then subtracted from the volumes determined on postchallenge images.

NIRF Imaging

For fluorescence excitation, three laser diodes operating at 660 nm with a power of 10 mW/cm² were used. The fluorescent light emitted from the lung samples was detected by a charge-coupled device (CCD) camera (Hamamatsu Photonics, Schüpfen, Switzerland) equipped with a focusing lens system (macro lens 60 mm, 1:2.8, Nikon). The CCD features 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 filter was used for selecting the wavelength at detection (700 nm). Data acquisition (i.e. integration) times ranged from 0.5 to 2.0 seconds depending on the intensity of fluorescence. The experiment was controlled by a PC using the Siemens SYNGO® software, which was used as well for quantitative analysis of the fluorescence images on a region of interest (ROI) basis.

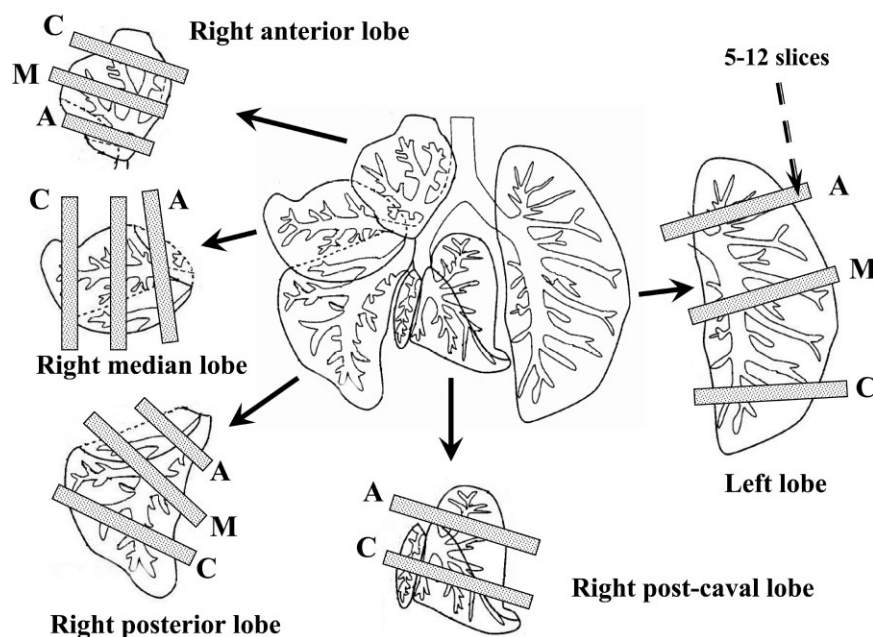


Figure 1. Diagram indicating the lobes of the rat lung and how they were sectioned for histologic analysis. Edema was assessed by histology on the apical (A), medial (M), and caudal (C) portions of each lobe (except for the postcaval lobe). In each lobe portion, 5–12 sections were analyzed, as indicated by the dashed arrow. Therefore, for each animal, between 15 and 36 sections were examined in each lobe.

From the quantified values of intensity and area where signals were detected, the fluorescence signal distribution was then calculated:

% of signal distribution for each individual

$$\text{lung lobe} = \frac{(\text{intensity} \times \text{area})}{\sum_{\text{all lobes}} (\text{intensity} \times \text{area})} \times 100$$

Histology

Lungs were fixed by slow in situ inflation with approximately 5 mL of 10% phosphate-buffered neutral formalin, pH 7.2, administered via a trachea cannula. After removal from the thorax, lungs taken from animals challenged with OVA remained in phosphate-buffered neutral formalin for a maximum of 72 hours. After fixation, lungs were trimmed and sections cut longitudinally through the left lobe and transversally through the four right lobes so as to include the main bronchi as well as the pulmonary alveoli. After processing to paraffin wax, both sections were embedded in blocks. Slices of 3- μ m thickness were cut from these blocks and then stained using the Verhoeff reaction. Perivascular edema was quantified on apical, medial, and caudal portions of each lobe (except for the postcaval) on slices captured at $\times 50$ magnification, following the scheme presented in Fig. 1. For each lobe portion, between 5 and 12 slices were examined with a light microscope (DMR BE; Leica Microsystems, Glattbrugg, Switzerland) connected to a video camera (ProgRes/3008; Jenoptic LOS, Eching, Germany). Morphometric analyses were performed with the image analysis software Image Access 3.10 (IMAGIC, Glattbrugg, Switzerland). The perivascular edema was determined as the difference between the external edema area and the external lamina elastica area, both circumscribed manually. Measurements were restricted to vessels with

diameters ranging from 80 to 500 μ m. Histologic perivascular edema (HPE) on each lobe corresponded addition of assessments performed on 15 to 36 slices (5–12 sections at the apical, medial, and caudal portions of each lobe). Scaling parameters, reflecting the relative size of each lobe (considering the total lung volume as 1), were used as weighting factors when computing the percentile distribution of edema between the lobes. The scaling parameters, determined from visible images obtained with the bonSAI small animal imager prototype, were the following (mean $\pm$ standard error of the mean [SEM] for eight animals): left lung lobe 0.34 ± 0.02 ; right apical lobe 0.17 ± 0.01 ; right median lobe 0.14 ± 0.01 ; right posterior lobe 0.20 ± 0.01 ; right postcaval lobe 0.15 ± 0.01 . With these scaling parameters, the areas of perivascular edema measured by histology were extrapolated into volumes. Thus, the total histologic edema volume for an animal was then estimated by adding the HPE for each lobe multiplied by the corresponding scaling parameter of the same lobe. The percentile edema distribution between the lobes was computed from the ratio between HPE for each lobe multiplied by the corresponding scaling parameter and the total histologic edema volume. The distribution of edema within a lobe was calculated from the ratios between the added values for each portion (apical, medial, or caudal) and the HPE of the lobe.

Statistics

Analysis of variance (ANOVA) multiple comparisons (Student-Newman-Keuls method) were carried out and significance was assumed at the 5% probability level.

RESULTS

Edematous signals were detected by MRI in all allergen-challenged rats. For instance, Fig. 2a shows axial im-

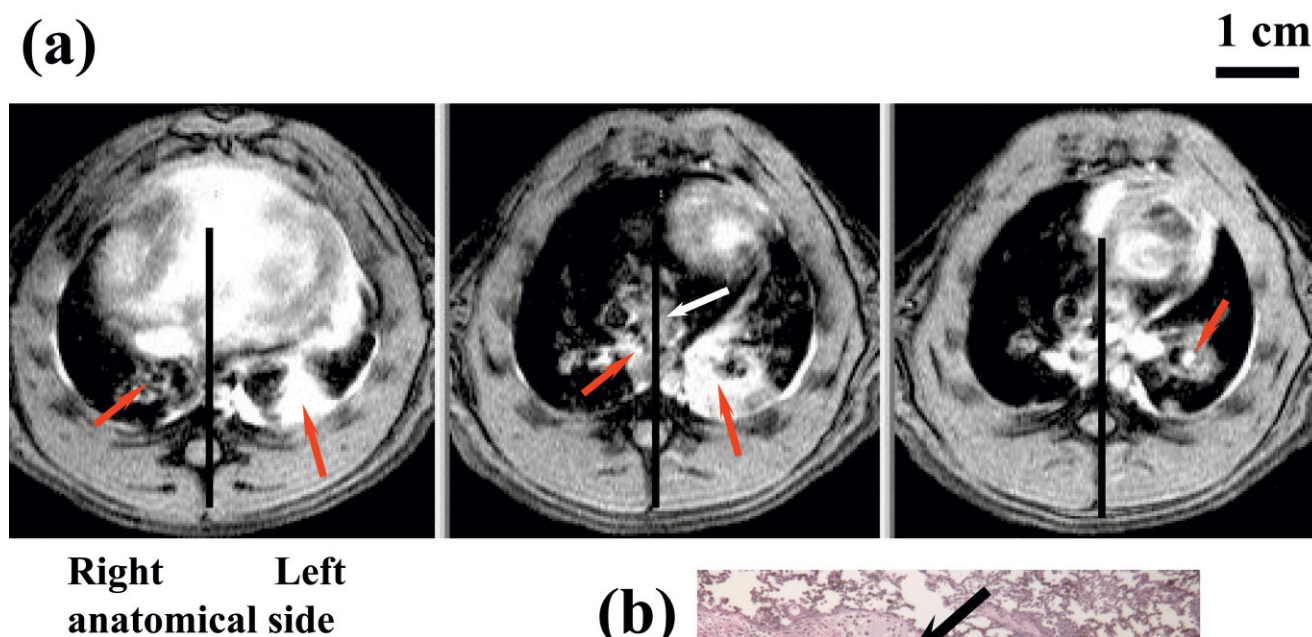


Figure 2. **a:** MR image through the thorax of a sensitized BN rat, acquired 24 hours after IT challenge with OVA. Edematous signals (red and white arrows) are seen prominently on the left anatomic side. The white arrow indicates edematous signals likely contributed by the right postcaval lobe. The animal breathed spontaneously, and neither respiratory nor cardiac gating was applied during image acquisition. For each rat, the volume of edematous signals was quantified on 16 consecutive slices covering the whole lung. **b:** Corresponding histologic section from the left lung of the same animal, demonstrating perivascular edema (black arrows).

ages from the thorax of an actively sensitized BN rat, 24 hours after IT administration of OVA. Strong and continuous edematous signals are prominently present at the left side of the body (continuous arrow) despite the fact that the allergen had been instilled before the bifurcation of the trachea, which would lead one to expect an equal fluid signal content on the left and right sides. In six out of eight rats edematous signals were more prominently seen on the left side of the body, whereas on the remaining two signals they were evenly distributed. As the individual lung lobes cannot be distinguished in the image and due to partial volume artifacts, it is likely that part of the edematous signal (e.g., that shown by the dashed arrow) appearing on the left anatomic side has indeed been contributed by the right postcaval lobe. The histologic section in Fig. 2b, obtained from the left lobe of the lung of the same animal, demonstrates marked perivascular edema.

The distribution of edema in the different lung lobes was studied by histology following the scheme presented in Fig. 1. Except for the postcaval lobe, edema volume was assessed in the apical, medial, and caudal portions of each lung lobe. Perivascular edema quantified by histology in each lobe of six actively sensitized BN rats 24 hours after allergen challenge is summarized in Fig. 3a. Whereas about 50% of the edema was found on the left lobe, the distribution of edema in the right lobes ranged between 9% (postcaval lobe) and 18%

(caudal lobe). By comparison, assessment of the volume of edematous signals by MRI on the same rats revealed that $56.2 \pm 2.8\%$ ($P = 0.006$) of the signals were localized on the left anatomic side of the animals. These results suggest that edema in the postcaval lobe also contributes to the MRI edematous signals observed on the left anatomic side, as mentioned above. The relative distribution of edema in the apical, medial, and caudal portions of each lung lobe (except for the postcaval) is summarized in Fig. 3b. Because of the small structure of the postcaval lobe, it was possible to cut it in two different portions only. Within the right anterior and median lobes the edema distribution was even, while a tendential gradient was observed within the other lobes (significantly decreased edema was found in the apical portion of the right posterior lobe only).

Next, we studied with NIRF imaging the pulmonary distribution of the fluorescent dye Cy5.5 directly instilled into the trachea using a similar procedure as that adopted for the challenge with OVA. A representative image from the extracted lungs of an animal that had received an IT instillation of the fluorescent dye is shown in Fig. 4a. Significant signal intensity differences were observed between the right and left side of the lungs. Nevertheless, it was not possible to precisely quantify the distribution of the dye in each individual lobe. Therefore, additional NIRF images of the separate lung lobes were taken (Fig. 4b). Marked and diffuse

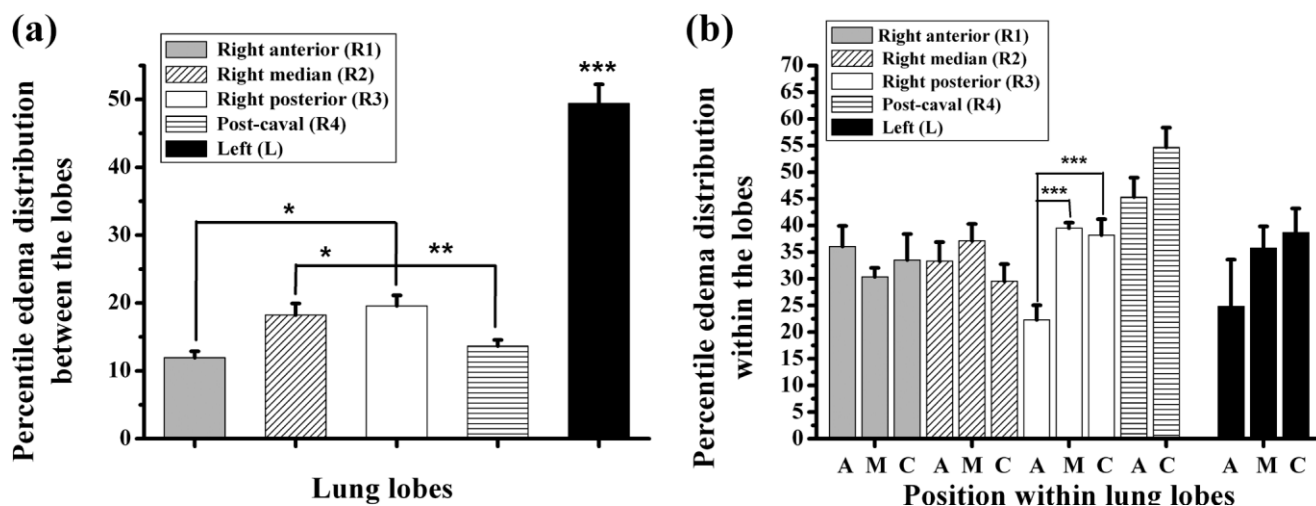


Figure 3. **a:** Percentile distribution of perivascular edema (means $\pm$ SEM; $N = 8$ rats) quantified by histology in each individual lobes of lungs taken from actively sensitized BN rats 24 hours after OVA challenge. The significance levels $* 0.01 < P < 0.05$, $** 0.001 < P < 0.01$, and $*** P < 0.001$ refer to ANOVA comparisons between the individual lobes. Edema was highly significantly elevated ($P < 0.001$) in the left lobe compared to all other lobes. **b:** Edema distribution (means $\pm$ SEM, $N = 8$ rats) among the apical (A), medial (M), and caudal (C) portions, determined by histology for each individual lobe (except for the post-caval). The level of significance $*** P < 0.001$ corresponds to ANOVA comparisons between different portions of the lobe.

fluorescence signals were essentially observed in the left lobe. Fluorescence was also observed in the right lobes but the intensity was significantly weaker and the signal more localized. The mean fluorescence signal distribution of Cy5.5 in the different lung lobes of actively sensitized BN rats is presented in Fig. 4c. Because a negligible fluorescence signal was detected in the trachea (Fig. 4a), it was not taken into account when calculating the signal distribution of the dye. Similarly to the edema distribution between the lobes after allergen challenge (Fig. 3a), the distribution of the dye particles was strongly uneven, with a significantly marked deposition in the left lobe when compared to that in the right lobes (Fig. 4c). However, the distribution was quite even between the four right individual lung lobes (range 9–14%). In addition, the distribution of the dye within the apical, medial, and caudal portions of each lobe (Fig. 4d) was remarkably similar to the distribution of the edema within the lobes (Fig. 3b). Within the right anterior and median lobes the edema distribution was even, while gradients were observed within the right postcaval and the left lobes. A significant gradient from apical to caudal was observed within the right posterior lobe.

DISCUSSION

The present study aimed at addressing the issue concerning the uneven distribution of edematous signals in the lungs of actively sensitized BN rats following IT allergen challenge (1–3). Extensive histologic analysis of perivascular edema confirmed the MRI observations, showing that allergic pulmonary inflammation in this model is observed primarily in the left lobe, with significantly lower signs of inflammation present in the other lobes. Overall, these results confirm previous studies showing that the MRI signals after allergen challenge primarily reflect perivascular edema (3). For pharmaco-

logic research, the importance of this finding lies on the fact that a variety of new compounds for the treatment of respiratory diseases are designed as antiinflammatory therapies. Because edema is an integral component of experimental pulmonary inflammation, MRI thus provides a noninvasive means of monitoring the course of the inflammatory response and the consequence of therapy with antiinflammatory drugs (4).

A possible relationship between deposition of an IT instilled fluorescent dye and the distribution of allergen leading to development of allergic pulmonary inflammation in the lungs of actively sensitized BN rats was also investigated. We explored the use of a biocompatible NIRF imaging agent, Cy5.5 (12). NIRF imaging allowed the monitoring of the biodistribution of the dye with a high temporal and spatial resolution. The specific feature of Cy5.5 is that it absorbs (670 nm) and emits (700 nm) at wavelengths at which blood and tissue are relatively transparent, and has high quantum yields.

Analogous to the MRI and the histological observations, the NIRF imaging data revealed that IT instillation of Cy5.5 led to a nonuniform distribution of the fluorescence signal, the left lobe having received most of the dye. The right median lobe received relatively less dye, which was in good agreement with other studies (6,9). The percentage of lung tissue area in contact with the dye was tendentially higher (albeit not significantly) for the apical lobar regions than the medial and the distal regions for the postcaval lobe (Fig. 4b). However, the gradient of distribution was reversed for the right posterior and the left lobes. The distributions within the three lobar regions for the right anterior and median lobes were rather uniform. These results are partially in agreement with previous studies. Leong et al (9) showed similar gradations in particle deposition for the posterior, anterior, and postcaval lobes of the rat, whereas a reverse distribution was seen for the left lobe. In another study, rats receiving IT instillations presented

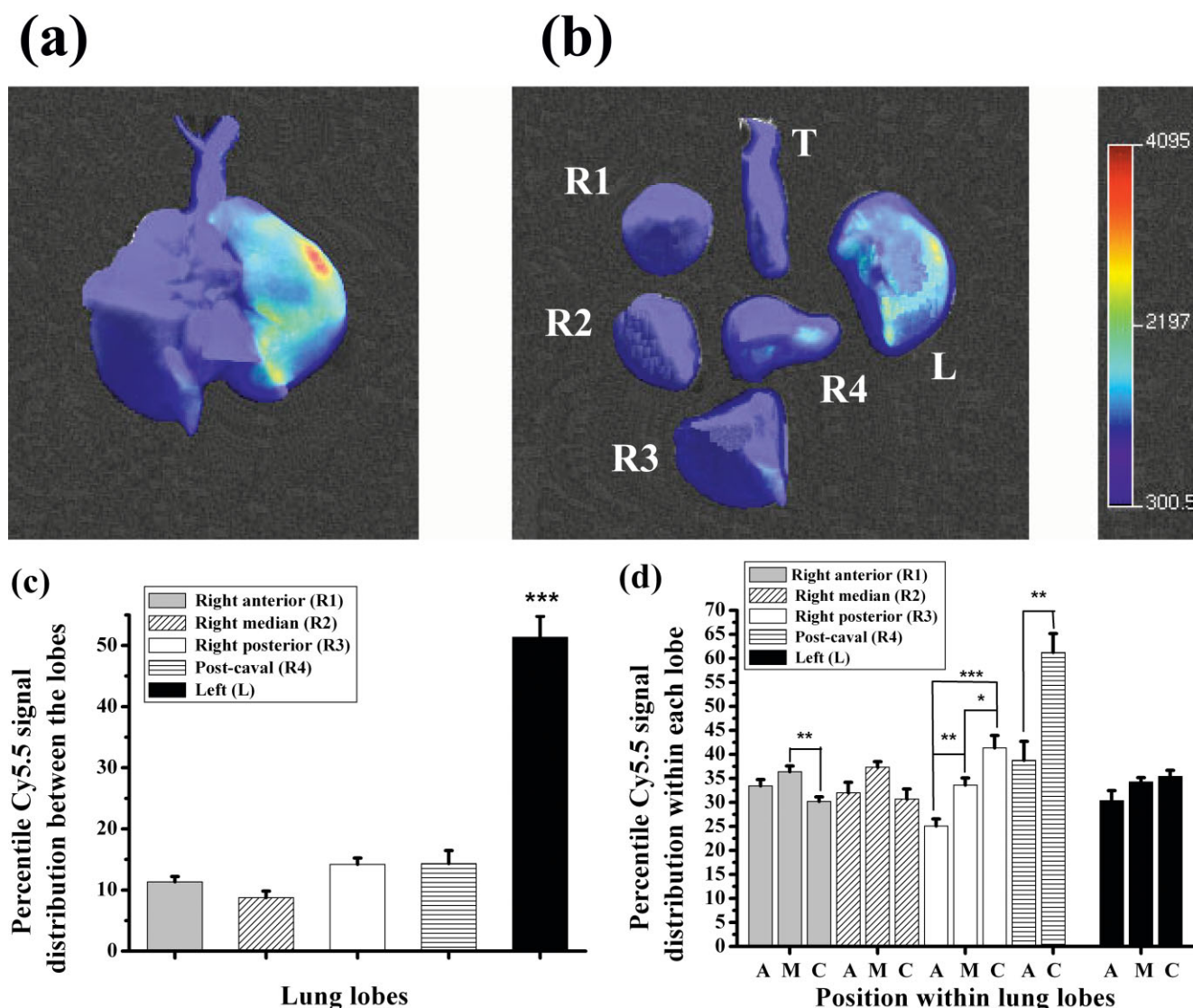


Figure 4. Representative NIRF images of a whole lung (a) and the individual lobes (b) taken from an actively sensitized BN rat after IT instillation of Cy5.5 (0.001% in PBS, 0.2 mL). The lungs were removed within the first hour after instillation of Cy5.5. **c:** Percentile distribution of fluorescence signal distribution (means $\pm$ SEM, $N = 8$ rats) determined in the individual lung lobes of BN rats after IT instillation of Cy5.5. Negligible fluorescence signal was detected in the trachea. R1: right anterior lobe; R2: right median lobe; R3: right posterior lobe; R4: right post-caval lobe; L: left lobe. The fluorescence signal in the left lobe was highly significantly elevated ($***P < 0.001$, ANOVA comparisons) with respect to the signals in the other lobes. **d:** Fluorescence signal distribution (means $\pm$ SEM, $N = 8$ rats) among the apical (A), medial (M), and caudal (C) portions of each individual lobe of the same animals. The significance levels * $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, and *** $P < 0.001$ refer to ANOVA comparisons carried out between the apical, medial, and caudal portions for each individual lobe.

larger deposition in the caudal portions of each lobe of the lung and less in the apical regions (6). One explanation for the difference in the lobar distribution may be the use of different methods of evaluation. In our study, we performed a planar evaluation of the dye distribution based on the intensity and area of the fluorescence signal detected by NIRF, whereas in the aforementioned studies, a histologic analysis of lung sections has been done. Other possible explanations for the observed discrepancy in the biodistribution would be the volume of the instilled carrier fluid and/or the time that the rats stay in a supine position on a 45° slanting animal-holding wire screen after IT instillation. Nevertheless, despite these differences within individ-

ual lobes, we and others demonstrated a predominant distribution of instilled material in the left lobe when compared to the right lobes.

The graphs showing perivascular edema quantified by histology and the fluorescent dye Cy5.5 distribution detected by NIRF in the different lung lobes presented a remarkably similar shape. The obvious conclusion from these data is that localized development of allergic pulmonary inflammation in actively sensitized BN rats is a consequence of the biodistribution of OVA in the lungs after IT instillation. However, because Cy5.5 and OVA have different molecular weights (1 and 45 kDa, respectively) and size (calculated distance from the left end to right end of the molecule is 20 and 67 Å, respectively),

the question arises whether the biodistribution of IT instilled material is dependent on the molecular size. Brain et al (6) demonstrated that changing particle size had only a small effect on the pulmonary distribution of IT instilled particles. Their results showed that the distribution of IT instilled particles was primarily determined by the properties and the volume of the carrier fluid rather than by the properties of the particles. Because in our experiments Cy5.5 and OVA were both diluted in 0.9 w/v saline and the volume instilled was the same, it is therefore reasonable to assume that Cy5.5 and OVA were similarly distributed in the different lung lobes, which was also reflected in the similarities of the NIRF imaging and the histology perivascular edema data. Overall, these remarks suggest that the distribution in the lung of a compound applied IT can be potentially followed noninvasively in vivo using NIRF imaging, by binding Cy5.5 to the drug. Although our measurements were carried out ex vivo using a two-dimensional projection technique, recent developments allow tomographic imaging data to be obtained from living small rodents using NIRF (13). Tomographic NIRF imaging may eventually allow one to improve the determination of the dye distribution within the lobes.

The question arises as to the reasons for the differential lobar particle distribution leading to uneven development of pulmonary inflammation. This difference in the distribution after IT instillation may be due to the inherent geometry of the bronchus of each lobe with respect to the left and right primary bronchi. A model of the lung airways of the rat has been developed from complete measurements of the tracheobronchial airways of a rat lung cast (8). Airway structural parameters such as segments, lengths, diameters, branching angles, and angles of inclination to gravity, all of which may influence the distribution of instilled material, were assessed. The authors assumed that the lobar weight of a lung was proportional to the number of terminal bronchioles in that lobe, leading to the conclusion that the bronchoalveolar tree was more developed in the left lobe. A similar conclusion was drawn in another study where it has been shown that the elastin content was significantly higher in the left lobe (14). Interestingly, an asymmetry in T_2^* relaxation times, possibly related to a more prominent bronchial tree on the left lung (15), has also been observed between the left and right lungs (16,17).

Histologic assessments can usually provide only a limited picture, albeit at great spatial resolution. In the present experiments, edema assessments were performed on 15–36 slices per lobe, and 5–12 slices were cut respectively on the apical, medial, and caudal portions of the lobes. With this relatively high number of assessments, and using scaling parameters reflecting the relative size of each lobe, we suppose that the provided values reflect quite well the edema distribution within and between the lobes. MR images covering the whole lung on the other hand, despite the lower spatial resolution, yield total edema volumes but not in the individual lobes, which cannot be discriminated by the technique.

In conclusion, we showed that the distribution of the IT instilled fluorescent dye Cy5.5 assessed by NIRF may

help to understand the uneven development of pulmonary inflammation in the rat lungs induced by allergen challenge and detected as edematous signal by MRI. Histologic quantification of perivascular edema confirmed that MRI primarily reflects what happens at the tissue level. MRI and NIRF imaging have the potential to provide important information for the preclinical pharmacologic research in the area of airway diseases. Because of their different nature, both techniques are complementary. MRI provides spatial resolution, but it lacks sensitivity; NIRF imaging has limited spatial resolution, of the order of 1.5 mm (18), but has a very high sensitivity, being capable of detecting substances in nanomolar concentrations. Thus, MRI is suited to address effects of compounds, for instance on the inflammatory response in models of airways diseases (4), but is not sensitive enough to detect the drugs themselves. Because of its sensitivity, NIRF imaging on the other hand may provide important insights concerning drug distribution and interaction in the lung, being thus suited for molecular imaging studies.

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