Identification with MRI of the pleura as a major site of the acute inflammatory effects induced by ovalbumin and endotoxin challenge in the airways of the rat

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Asthma and chronic obstructive pulmonary disease (COPD) are chronic disorders of the lung that involve the interplay of many inflammatory and structural cells, resulting in the interaction of a wide variety of inflammatory mediators that include auto-}

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MATERIALS AND METHODS

Experiments were carried out with the approval of the Veterinary Authority of the City of Basel (license no. 567). Animals. Forty-eight male BN rats (Iffa Credo, L’Arbresle, France) weighing 250–300 g were used in the study. Animals were kept at an ambient temperature of 22 ± 2°C under a 12-h normal phase light-dark cycle and fed with NAFAG pellets (Nahr und Futtermittel AG, Gossau, Switzerland). Drinking water was freely available.

Sensitization. Twenty-four rats were sensitized to OA using the following standard protocol (see Ref. 16): OA (20 μg/ml; n = 12) or saline (0.2 ml; n = 12). Naïve rats received either LPS (1 mg/kg; n = 12) or saline (0.2 ml; n = 12). Substances were administered intratracheally through a cannula positioned above the carina.

MRI. For MRI, rats were anesthetized with 2% isoflurane in a mixture of O2/N2O administered via a face mask. Animals were placed in supine position in a cradle made of plexiglas. Body temperature was maintained at 37 ± 1°C using warm air regulated by a rectal temperature probe (DM 852, Ellab, Copenhagen, Denmark). During examinations, animals breathed spontaneously and neither cardiac nor respiratory triggering was applied.

Measurements were performed using a Biospec 47/40 spectrometer (Bruker Medical Systems, Karlsruhe, Germany) operating at 4.7 T, equipped with an actively shielded gradient system capable of generating a gradient of 200 mT/m. The operational software of the scanner was Paravision (Bruker). For detection of fluid signals, a T1-weighted, gradient-echo sequence with the following parameters was used: repetition time, 5.6 ms; echo time, 2.7 ms; flip angle of the excitation pulse, ~15°; field-of-view, 6 × 6 cm2; matrix size, 256 × 128; and slice thickness, 1.5 mm. A single slice image was obtained by computing the 2-dimensional Fourier Transformation of the averaged signal from 45 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 59 s for a single slice. The entire lung was covered by 18 consecutive transversal slices. The duration of a session, including anesthetizing and positioning the animal, did not exceed 25 min.

MRI analysis. The volume of fluid signal was quantified using a semiautomatic segmentation procedure implemented in the IDL (Interactive Data Language Research Systems, Boulder, CO) environment (version 5.1) on an SGI O2 (Silicon Graphics, Mountain View, CA) system. Images were first weakly low-pass filtered with a Gaussian profile filter and then transformed into a set of four gray level classes using adaptive Lloyd-Max histogram quantization. This method avoids operator bias due to the arbitrary choice of threshold levels on each image (8). Because the fluid comprised high signal intensities in the original images, it was represented by the highest gray level class in the transformed images. This class could be extracted interactively by use of a region grower. Because of the unknown extent of the fluid, no morphology parameters were incorporated into the region-growing process. Instead, a contour serving as an unknown extent of the fluid, no morphology parameters were incorporated into the region-growing process. Instead, a contour serving as a growing border was drawn to control region growing manually. The segmentation parameters were the same for all the analyzed images, chosen to segment regions corresponding to high intensity signals. Because the edematous signals and those from 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. For the edematous responses of the pleura, signal volumes were determined at time points of 3 and 6 h after challenge with OA, LPS, or saline; no baseline measurements were taken due to the lack of MRI-visible vessels at the pleural region.

Postmortem analyses. Rats were killed by an overdose of pentothal (250 mg/kg ip) administered 6 h after challenge and immediately following MRI signal acquisition.

Histological analysis. Lungs were inflated with 5 ml of 10% phosphate-buffered neutral formalin via a cannula inserted in the trachea. The lungs were then removed from the thorax and immersed in formalin between 24 and 72 h. The lung tissue was then sectioned transversally through the left lobe, the right apical, the right median, and the right caudal lobes to include the main bronchi as well as the pulmonary alveoli. After we processed to paraffin wax, sections were embedded in blocks. Slices of 3-μm thickness were cut from these blocks and then stained using Alcian blue-periodic acid-Schiff (PAS) stain for the determination of peribronchial edema and detection of goblet cells and acidic and neutral mucus. Staining using Verhoeff’s reaction was performed for quantification of perivascular edema.

Determination of pleural edema. Two pictures of the left lobe and two pictures of the right caudal lobe of each animal were taken at the level of the hilus so that the visceral pleura (that closely covers the lung) and the parietal pleura (that is adherent to the thoracic wall and the diaphragm) were present. Pleural edema was defined as the presence of increased fluid in the pleural cavity (the space between the parietal and the visceral pleurae). For the quantification of pleural edema, the distances between the visceral and the parietal pleura at four different levels were determined.

Quantification of peribronchial edema. Three to four pictures of bronchi from each section of the left lobe (apical, median, and caudal) were captured at ×10 magnification on PAS/Alcian blue-stained slides. Morphometric analyses were performed with the software Image Access 4.0 (Imagic, Glatbrugg, Switzerland) connected to a video camera Prog/Res/3008 (Jenoptik LOS, Eching, Germany). The areas of smooth muscle layer and peribronchial edema were manually circumscribed, and peribronchial edema was calculated as a percentage of smooth muscle layer area.

Quantification of perivascular edema. Five to eight pictures of arteries from each section of left, right caudal, and right median lobes were captured at ×10 magnification on Verhoeff-stained slides. As before, morphometric analyses were performed with the software Image Access 4.0. The areas of external edema and external elastica lamina were manually circumscribed, and perivascular edema was calculated as a percentage of external elastica lamina area. Only vessels with internal diameters between 35 and 150 μm were measured. Approximately 25 vessels were assessed per animal. The total edema determined histologically (sum of the edema in the left, right caudal, and right median lobes) is shown as a percentage.

Quantification of goblet cells. Three to seven pictures of bronchi from each section of left, right caudal, and right median lobes were captured at ×10 magnification on PAS/Alcian blue-stained slides. The circumference of each bronchus was manually delineated, and the number of goblet cells was counted. Results were expressed as the number of goblet cells per millimeter length of bronchi. Only bronchi whose diameters comprised between 900 and 2300 μm were measured. The total goblet cell numbers (sum of those in the left, right caudal, and right median lobes) are presented.

BAL fluid analysis. A detailed description of the BAL procedure and the analysis of the parameters of inflammation in the BAL fluid have been provided earlier (10, 36). Briefly, after killing the animal with an overdose of pentothal, the lungs were lavaged. For leukocyte numbers and cell differentiation, an automatic cell-analyzing system was utilized (Cobas Helios 5Diff, Hoffmann-La Roche, Axon Laboratory). Determination of eosinophil peroxidase was based on the oxidation of O-phenylenediamine by eosinophil peroxidase in the presence of hydrogen peroxide. Myeloperoxidase (MPO) activity was measured in a photometric assay based on the oxidation of O-dianiside dihydrochloride by MPO in the presence of hydrogen peroxide. The level of protein in the BAL fluid supernatants was measured.
Fig. 1. A: axial sections through the thorax of an actively sensitized rat treated with saline (left) or 0.3 mg/kg intratracheally of ovalbumin (OA; middle) and of a naïve rat treated with 1 mg/kg intratracheally of LPS (right) acquired 6 h after challenge. The arrows show the appearance of pleurisy in OA- and LPS-treated animals. B: pleural fluid signal volumes determined from MRI acquired at 3 and 6 h after challenge in sensitized rats treated with saline (0.2 ml intratracheally) or OA (0.3 mg/kg intratracheally) and in naïve rats treated with saline or LPS (1 mg/kg intratracheally). Values are means ± SE. ***P < 0.001 and **0.001 < P < 0.01 show that the value differs significantly from the value in the respective vehicle-treated group. $0.001 < P < 0.01$ shows that the MRI signal volume 3 and 6 h after OA challenge in sensitized rats differs from each other. #0.001 < P < 0.01 and ###P < 0.001 refer to differences between OA and LPS at 3 and 6 h after challenge, respectively. C: pleural edema assessed as the distances (means ± SE) between the visceral and parietal pleura from the left and right caudal lobes. The distance between challenged rats was significantly greater (**P < 0.01; Student’s t-test) than that in corresponding saline-treated animals. There was a significant difference between OA- and LPS-treated animals (#P < 0.05). D: histological sections of the left lobe of naïve rats or actively sensitized rats, 6 h after challenge. The arrows show the edematous response elicited by allergen and endotoxin in the pleural cavity. The number of animals per group was 6; samples were analyzed with the Student’s t-test (1 tail) for B and C. Sens., sensitized.
measured by a photometric assay, based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Mucus in BAL fluid was assessed using the sandwich enzyme-linked lectin assay as described earlier (10) by measuring the optical density of flat-bottomed Costar plates coated with Ulex europaeus agglutinin-1 (Sigma) at a wavelength of 492 nm using a SpectraMax 250 plate reader (Molecular Devices, Surrey, UK).

Statistics. Student’s t-test (1 tail) was performed for MRI, BAL fluid, and histological data referring to edema. The Mann-Whitney’s U-test was chosen for analysis of histological data relating to goblet cell numbers.

RESULTS

Six hours after challenge with OA, MRI images revealed a prominent edematous signal at the edges of the lung corresponding to the pleura. A similar although less intense signal was seen in LPS-treated rats (Fig. 1A). Determination of pleural edema signal volume by MRI showed a significantly greater response in OA- than LPS-treated rats (Fig. 1B). Histological assessment of the pleural cavity revealed a more pronounced pleurisy in the OA compared with the LPS group (Fig. 1, C and D).

At the early time points of 3 and 6 h after instillation of OA or LPS, irregular fluid signals of similar appearance were apparent in MRI of the lung (Fig. 2A). However, OA treatment resulted in higher levels of fluid volumes than LPS challenge at both time points (Fig. 2B). Postmortem BAL fluid analysis, performed 6 h after challenge, revealed greater increases in protein concentration and markers of cellular activation (MPO) and eosinophil peroxidase (EPO) in OA-treated animals compared with the LPS-treated group, although greater inflammatory cell recruitment (macrophages, eosinophils, and neutrophils) was observed in the LPS-treated rats (Table 1). No increased mucin levels were detected using the lectin enzyme assay in BAL fluid samples from either group (Table 1). Increased levels of peribronchial and perivascular edema were determined by histology at 6 h after OA or LPS compared with saline instillation. Furthermore, whereas OA and LPS led to comparable levels of perivascular edema, peribronchial edema was significantly higher in OA-challenged lungs (Fig. 2, C and D). No significant differences in goblet cell numbers were observed between OA or LPS and the corresponding saline-challenged animals; however, actively sensitized rats presented higher levels of goblet cells than naïve animals (Fig. 2E).

DISCUSSION

Perhaps the most striking finding in the present study was the appearance of a marked MRI signal arising from the pleura 6 h after OA and a signal of lower magnitude following LPS challenge (Fig. 1, A and B). The MRI signal correlated with histological analysis revealing severe edema in the pleural cavity of OA-treated animals. LPS rats scored slight edema, and no abnormality was observed in saline- (vehicle-) treated, sensitized, or naïve rats (Fig. 1, C and D). The pleura is a target site for toxic effects induced by a variety of particles (15). A high density of mast cells is distributed throughout the lung pleura in rodents (37). Models of antigen-induced pleurisy based on intrapleural injection of OA in sensitized rats have been described (4, 33, 27). Furthermore, it has been shown in vitro that pleural mast cells are important contributors to histamine and leukotriene release (38). In vivo studies performed by Nagase et al. (30) in BN rats showed that 5-hydroxytryptamine (5-HT) and leukotriene (LTD4), both known to be released by mast cells (14), are involved in the early response to OA in sensitized animals. Experiments in actively sensitized rats have also shown that intrathoracic OA administration (to the pleural cavity) resulted in an increase of leukocytes and protein concentration in the pleural cavity as early as 30 min after challenge, which was still detected 4 h later (13, 26).

Because of its innate close proximity to the lung, the pleura is prone to react to inflammatory events occurring in the lung parenchyma (2). Changes in pleural permeability leading to exudative pleural effusions with high protein content have been observed in local or systemic diseases (24). Exposure of pleural mesothelial monolayers to agents such as thrombin, LPS, or bacteria can induce changes in pleural permeability to proteins (1, 22). Thus it is plausible that local parenchymal inflammation induced by intratracheal OA or LPS challenge may lead to a disruption in pleural permeability, resulting in an increase of fluid in the pleural space. Moreover, migration of neutrophils, mononuclear phagocytes, and lymphocytes from the vascular compartment to the pleural space occurs during inflammation (12, 29, 34). Therefore, the migration of activated inflammatory cells to the pleural space following OA challenge may contribute further to allergen-induced pleurisy.

Intratracheal instillation of OA or LPS resulted in the appearance of similar irregular MRI fluid signals at 3 and 6 h after challenge with either substance. A larger fluid signal...
volume was observed in allergen-treated animals. These MRI observations correlated with the determination of perivascular edema by histology 6 h after challenge (Fig. 2, A–C). MRI detects the overall presence of edema, but due to limited spatial resolution, it is unable to discriminate between the perivascular and peribronchial components (8, 9). Early after challenge, the levels of perivascular edema were similar in the OA and LPS groups, but peribronchial edema was accentuated following allergen. This explains the increased MRI fluid signal volume in OA- compared with LPS-treated animals. However, at later time points, e.g. 24 or 48 h after challenge, the major contribution to the MRI signals may originate from perivascular edema (36).

BAL fluid analysis showed an increase of inflammatory parameters in both OA- and LPS-treated animals 6 h after challenge. LPS caused a greater influx of inflammatory cells compared with saline treatment, whereas the latter manifested increased levels of markers of inflammatory cell activation (EPO and MPO) and protein concentration in the BAL fluid, 2) no significant mucin was detected in the BAL, and 3) there was no significant increase of goblet cells or flocculent material in histological sections, indicating that the signals observed 3 and 6 h after challenge were most likely due to edema and not increased mucus production. Nevertheless, an increased goblet cell number was observed in sensitized animals compared with naïve animals. Such differences between sensitized and naïve animals have been described previously (32). Thus it is likely that early after challenge, OA and LPS activate a pathway that translates into the induction of a weak edematous response that develops further over 24 h in the OA model (8) and that precedes mucus release induced by LPS (10) observed at later time points.

The mucus-like appearance of the MRI lung edematous signals at 3 and 6 h following OA or LPS could possibly be explained by local magnetic field inhomogeneities produced by differences in magnetic susceptibilities between the lung tissue and air that comprise ~80% of the pulmonary volume, resulting in very short T2* relaxation times [of the order of 500 μs at 4.7 T (8)]. The present results suggest that for low levels of edema such as those present at these early points after challenge, the lung characteristics influence the T2* relaxation mechanism of the water. In other words, the presence of low fluid volumes in the lung can result in a signal appearance that does not allow a discrimination between edema and mucus. This is also the case when low volumes of saline are administered intratracheally, leading to signals of mucus-like appearance that disappear typically within 30 min following instillation. At later time points, e.g., 24 h following OA, the presence of marked edema ensures a relaxation mechanism dominated by free water that leads to a strong and uniform signal (8), thereby supplanting the influences of the lung characteristics on the signal behavior.

In summary, our results point to the opportunity of non-invasively studying with MRI the influence of pulmonary insults on inflammatory events in the pleura. Furthermore, histological analyses suggested that the early inflammatory responses following OA and LPS and detected by MRI in the lungs were primarily due to peribronchial edema.
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REFERENCES