Noninvasive detection of endotoxin-induced mucus hypersecretion in rat lung by MRI

NICOLAU BECKMANN,1 BRUNO TIGANI,1 ROSEMARY SUGAR,2 ALAN D. JACKSON,2 GARETH JONES,2 LAZZARO MAZZONI,3 AND JOHN R. FOZARD3

1Central Technologies and 3Respiratory Diseases Therapeutic Area, Novartis Pharma, CH-4002 Basel, Switzerland; and 2Novartis Horsham Research Centre, Horsham RH12 5AB, United Kingdom

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Beckmann, Nicolau, Bruno Tigan, Rosemary Sugar, Alan D. Jackson, Gareth Jones, Lazzaro Mazzoni, and John R. Fozard. Noninvasive detection of endotoxin-induced mucus hypersecretion in rat lung by MRI. Am J Physiol Lung Cell Mol Physiol 283: L22–L30, 2002.—Using magnetic resonance imaging (MRI), we detected a signal in the lungs of Brown Norway rats after intratracheal administration of endotoxin [lipopolysaccharide (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. Bronchialalveolar 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 major factor contributing at the later time points. The facts that animals can breathe freely during data acquisition and that neither respiration nor cardiac triggering is applied render this MRI approach attractive for the routine testing of anti-inflammatory drugs. In particular, the prospect of noninvasively detecting a sustained mucus hypersecretory phenotype in the lung brings an important new perspective to models of chronic obstructive pulmonary diseases in animals.

Chronic obstructive pulmonary disease (COPD) is a complex, multicellular disease in which specific and nonspecific factors result in bronchial obstruction and chronic inflammation (for a recent review see Ref. 29). COPD is a major cause of mortality and a significant drain on health care resources (9, 40). A critical component of the disease is inflammation of the pulmonary mucosa and submucosa, characterized by an infiltration of the airways with neutrophils. The major issues concerning COPD are prevention of the disease, slowing its progression once diagnosis has been established, and prevention, as well as more effective treatment, of exacerbation (41).

Animal models have been established in an attempt to mimic and study specific aspects of human respiratory disease (30). An inflammation similar to that observed in COPD patients can be elicited in animals with the administration of the endotoxin lipopolysaccharide (LPS), a bacterial macromolecular cell surface antigen. LPS activates mononuclear phagocytes through a receptor-mediated process, leading to the release of a number of cytokines, including tumor necrosis factor-α (TNF-α) (42, 43). TNF-α increases the adherence of neutrophils to endothelial cells, thus facilitating a massive infiltration of neutrophils into the pulmonary spaces (2).

The majority of animal studies involving endotoxins has been carried out in mice (8, 13, 27, 28), due in part to the ease with which lung injury can be induced by systemic LPS administration in this species, but also because monoclonal antibodies to many mouse cytokines are available. However, the Brown Norway (BN) rat, which is used extensively in the investigation of the pathophysiology of allergic asthma (12, 17, 18), is also a suitable animal in which to study LPS-induced pulmonary injury (22, 24, 35, 39). Exposure of rats to LPS is characterized by infiltration of the alveolar and bronchiolar air spaces by neutrophils (34) and induction of mucous cell metaplasia (19).

Recently, magnetic resonance imaging (MRI) was used to investigate noninvasively the development of an edematous signal in the lungs of actively sensitized BN rats after intratracheal allergen challenge (5). In the present study, a similar approach has been used to detect and quantify the signal generated in the lungs of BN rats after intratracheal instillation of LPS. Images were acquired at regular intervals ≤16 days postchallenge. MRI results were compared with the inflammatory status of the lung, represented by the degree of cell infiltration into the alveolar space, and the mucus concentration, determined by bronchoalveolar lavage (BAL) fluid analysis.

Materials and Methods

Animals. Male BN rats (Iffa-Credo, L’Arbresle, France) weighing ~250 g were used. They were housed in a temperature- and humidity-controlled environment and had free

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access to standard rat chow and tap water. All experiments were carried out according to the Swiss federal regulations for animal protection.

**LPS exposure.** Animals were anesthetized with 4% isoflurane (Abbott, Cham, Switzerland). LPS from *Salmonella typhosa* (0.03, 0.3 or 1 mg/kg dissolved in 0.2 ml saline; Sigma, Dorset, UK) or vehicle (0.2 ml of saline) was administered intratracheally, and the animals were allowed to recover.

**BAL.** A detailed description of the bronchoalveolar lavage (BAL) procedure and the analysis of the parameters of inflammation is provided in Ref. 5. Briefly, animals challenged with 1 mg/kg of LPS or saline were killed with an overdose of pentobarbital (250 mg/kg ip) immediately after an MRI examination. The lungs were lavaged, and the following parameters were assessed in the BAL fluid: number of macrophages, eosinophils, and neutrophils; myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activities, and TNF-α, and total protein and mucus content.

**Determination of rat TNF-α in BAL fluid.** Fifty microliters of the samples and standards, in duplicate, were added to the TNF-α antibody-coated wells. Fifty microliters of biotinylated anti-TNF-α (Biotin Conjugate) solution were pipetted into each well except the chromogen blanks. The plates were incubated for 1 h at room temperature and then washed four times. Streptavidin-horseradish peroxidase (HRP, 100 μl) was then added to each well except the chromogen blanks.

After incubation for 45 min at room temperature, the wells were washed four times, and 100 μl of stabilized chromogen were added to each well. The plates were incubated for 30 min in the dark at room temperature. The absorbance of each well was read at 450 nm. Results were expressed as picograms per milliliter, using a standard curve established with standards.

**Determination of mucus in BAL fluid using the sandwich enzyme-linked lectin assay.** Ninety-six-well, high-binding, flat-bottomed microtiter plates (Costar) were coated with 100 μl of *Ulex europaeus* agglutinin-1 (UEA-1, Sigma) at 1.25 μg/ml in coating buffer (35 mM sodium bicarbonate, 15 mM sodium carbonate, pH 9.5). Plates were covered with (sticky back film) and incubated overnight at 4°C. Excess lectin was removed by washing three times with 200 μl per well of wash buffer (10 mM PBS, 0.05% Tween 20, and 0.05% gelatin; PBS-T-G). Plates were tapped dry before the addition of blocking buffer (10 mM PBS, 0.1% Tween 20; 150 μl) and incubation for 1 h at 37°C. Plates were washed three times as above with PBS-T-G and either used immediately or stored at −80°C for up to 6 mo. Purified human mucus standard (100 μl; 24 μg/ml) was serially diluted in PBS over nine wells in duplicate. Samples that had been stored frozen at −80°C were thawed and added to the plates (100 μl) in duplicate. PBS was substituted for samples in six wells on each plate to serve as reagent controls. Plates were incubated for 1 h at 37°C, then washed four times with 200 μl of PBS-T-G. HRP-conjugated UEA-1 (UEA-1/HRP; Sigma or EY Laboratories) was added at 1.25 μg/ml in PBS (100 μl). Plates were incubated for 1.5 h at 37°C, and then each plate was washed six times with 200 μl of wash buffer. Substrate (0.05% orthophenylenediamine dihydrochloride; Sigma) in buffer (0.15 M citrate phosphate buffer, pH 5.0, with 0.015% hydrogen peroxide added immediately before use) was prepared in a foil-covered container and added to the plate (150 μl). The color development was measured at a wavelength of 492 nm using a SpectraMax 250 plate reader (Molecular Devices, Surrey, UK). A purified human mucus sample from an otherwise healthy smoker was used as a standard to convert optical densities to mucus concentrations. The gravimetric weight was used to assign the concentration of mucus in the standard. Approximately 80% of mucus consists of carbohydrate side chains, of which substantial amounts are α-L-fucose, which is detected by UEA-1 lectin. Because the epitope is present in such abundance, it can be used as a generic marker of mucus concentration.

The enzyme-linked lectin assay has been validated to detect high-molecular-weight material derived from goblet cells and submucosal glands with no reactivity against rat whole blood lysates, rat plasma, or lysates of rat BAL leukocytes. Lung sections incubated with UEA-1 stained rat goblet cells (and glands, where these were present) highly selectively, and there was no background staining of other tissue elements. As mentioned above, the assay detects α-L-fucose, which is present in mucus from both glands and goblet cells. Because the actual quantities of α-L-fucose may be different in mucins from these different sources, α-L-fucose equivalents rather than absolute mucin concentrations were actually measured.

**Histology.** Challenged rats were killed by an overdose of pentobarbital (250 mg/kg ip). Lungs were perfused in situ via a cannula inserted into the pulmonary artery with 30 ml of modified Krebs solution (composition in mM: 118 NaCl, 4.8 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 1.2 KH₂PO₄, 25 NaHCO₃, and 11 glucose) and inflated with −5 ml of 10% phosphate-buffered neutral formalin (BNF), pH 7.0, via the tracheal cannula. After being removed from the thorax, lungs were immersed in BNF for at least 24 h but not longer than 72 h. After 3 days of fixation, the lung tissue was dissected into 5-mm-thick slices and processed into paraffin wax overnight, before being embedded into a wax block. Sections of 4 μm thickness were cut and then stained with hematoxylin and eosin for general morphology or with Alcian blue-periodic acid Schiff for the detection of acid and neutral mucus and identification of goblet cells. For immunostaining, sections were placed in metal staining wax and passed through xylene and industrial methylated spirit. Slides were then treated with hydrogen peroxide in methanol to inhibit endogenous peroxidase and subsequently with 0.1% trypsin in 0.1% calcium chloride. Slides were then washed, and after draining, labeled lectin diluted with tri-buffered saline was applied, and slides were maintained overnight at 4°C. After washing, second-stage antibody (rabbit anti-FITC/HRP; DAKO) diluted in Tris-HCl (pH 7.6) was applied for 30 min. Slices were then again washed, and freshly prepared diaminobenzidine solution was applied for 10 min. After further washing, nuclei were counterstained in Coles hematoxylin. Goblet cells were quantified using a KS400 image analyzer (Imaging Associates, Thame, UK). The program produced a binary image of the microscopic field (from a video camera), detected the more darkly stained goblet cells from the negatively stained background, and created a 20-pixel-deep zone down from the apical surface of the epithelium (enough to include only the surface cells). All other areas of the field of view were ignored. The number and area of goblet cells present within this zone were then calculated.

**MRI.** Measurements were carried out with a BioSpec 47/40 spectrometer (Bruker, Karlsruhe, Germany) operating at 4.7 T. A gradient-echo sequence (14) with repetition time 5.6 ms, echo time 2.7 ms, band width 100 kHz, flip angle of the excitation pulse ~15°, field of view 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 transformation of the averaged signal from 60 individual image acquisitions and interpolat-
ing 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 s for a single slice. The entire lung was covered by 28 consecutive slices. A birdcage resonator of 7 cm in diameter was used for excitation and detection. During MRI measurements, rats were anesthetized with 2% isoflurane in a mixture of O2-N2O (1:2), administered via a face mask, and placed in supine position. The body temperature of the animals was maintained at 37°C by a flow of warm air. Total examination time per animal, including positioning, was ~40 min. The examination protocol for each animal consisted of acquiring a set of baseline images before the LPS challenge. Then, images were acquired at 1, 6, 24, 48, 72, 96, 144, and 192 h after the challenge.

*Magnetic resonance image analysis.* The volume of signals appearing in the lung after LPS challenge was determined by 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 quantitation (21). This method avoided operator bias due to arbitrary choice of threshold levels on each image. Signals in response to LPS were 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 signals detected in the lung, no morphology parameters were incorporated in 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. Be-
cause 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.

Statistics. Student’s t-test with the Bonferroni correction was applied using the saline-treated rats as control group.

RESULTS

MRI of rat lung after challenge with LPS. Figure 1A shows representative transverse sections through the thoracic region of a BN rat before and at various times after intratracheal exposure to LPS (1 mg/kg). Clear signal changes were present in the lung within a few hours after application of the endotoxin. The signals in response to LPS had two components. One was characterized by a diffuse signal and was particularly prominent until ~48 h after LPS challenge. A second component, characterized by an irregular appearance and much weaker signal intensity, was present in the first hours after LPS challenge but predominated at the later time points. It was not possible to differentiate the individual components, as they overlapped. Thus results are presented as total signal volume. No signal changes were seen in the lung at any time point after saline challenge (data not shown). For comparison, an axial section through the thorax of an actively sensitized BN rat, acquired 24 h after intratracheal challenge with ovalbumin (OA, 0.3 mg/kg), is shown in Fig. 1B (left). The intense and diffuse signal appearing in the lung after OA instillation is related to edema formation as has been described elsewhere (5). To illustrate the differences between edema and mucus as detected by MRI, test tubes containing 10 ml of either mucus (sputum obtained from a heavy smoker) or water were imaged with the same acquisition parameters as for the in vivo images (Fig. 1B, right). A clear difference was evident, which reflected the in vivo findings. Thus the sputum sample showed an irregular appearance, whereas the water sample was diffuse and of higher intensity.

The time course of the signal development in the lung after challenge with different doses of LPS is shown in Fig. 2. A dose-related response was observed, with the maximum signal enhancement occurring 6 h postchallenge at 0.03 and 0.3 mg/kg and between 24 and 48 h after 1 mg/kg of LPS. The signal was of long duration. For example, after the higher dose of LPS, a signal was still detected 8 days after dosing (Fig. 2).

Comparison between signal changes in the lung detected by MRI and BAL fluid parameters of inflammation after challenge with LPS. The time course of the response in the airways induced by intratracheal challenge with LPS (1 mg/kg) detected as an MRI signal in the lung was compared with the inflammatory status of the lungs defined by analysis of the BAL fluid (Table 1). Animals were killed at each time point immediately after the MRI acquisitions and the BAL fluid was recovered. In confirmation of the observations summarized in Fig. 2, challenge with LPS led to an extensive signal in the lung, with a peak of 0.79 ± 0.05 ml at 48 h. The signal declined by ~50% from this peak at 96 h but was still detectable 16 days after the administration of LPS. BAL fluid analysis revealed a marked increase in the number of neutrophils at 24 h after

Table 1. Comparison between signal detected by MRI at different time points with respect to LPS (1 mg/kg it) or saline challenges, and BAL inflammatory cell infiltration, protein and TNF-α concentrations, and EPO and MPO activities

<table>
<thead>
<tr>
<th></th>
<th>Saline Challenge</th>
<th>LPS Challenge</th>
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<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>MRI signal volume, ml</td>
<td>0.05 ± 0.023</td>
<td>0.31 ± 0.07†</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.76 ± 0.15</td>
<td>2.37 ± 0.29‡</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.76 ± 0.15</td>
<td>71.93 ± 14.83</td>
</tr>
<tr>
<td>Macrophages</td>
<td>4.96 ± 0.67</td>
<td>12.57 ± 2.39†</td>
</tr>
<tr>
<td>Protein</td>
<td>0.22 ± 0.01</td>
<td>0.43 ± 0.04‡</td>
</tr>
<tr>
<td>EPO</td>
<td>3.47 ± 0.32</td>
<td>11.8 ± 1.25‡</td>
</tr>
<tr>
<td>MPO</td>
<td>37.05 ± 10.26</td>
<td>362.13 ± 17.33‡</td>
</tr>
<tr>
<td>TNF-α</td>
<td>90.53 ± 20.29</td>
<td>246.03 ± 16.94‡</td>
</tr>
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Data are expressed as means ± SE for n = 6 rats. Eosinophils, neutrophils, and macrophages are expressed in 10⁶ cells/12 ml, eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activities in mU/ml, and protein and tumor necrosis factor (TNF-α) concentrations in µg/ml and pg/ml, respectively. Rats were killed at different time points immediately after magnetic resonance imaging (MRI) measurement, and bronchalveolar lavage (BAL) fluid was extracted. *P < 0.05, †P < 0.01, ‡P < 0.001; significance level of the difference between animals challenged with saline or LPS.

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LPS, which declined at later time points but was still significantly elevated at 384 h. Significant and sustained increases in macrophages, eosinophil number, and protein concentration were observed up to 192 h after LPS challenge. MPO and EPO activities were significantly elevated up to 48 h. TNF-α concentrations were significantly increased 24 h after challenge but fell below the levels in saline-challenged rats at the later time points.

Comparison between signal changes in the lung detected by MRI and the mucus concentration in the BAL fluid after LPS-challenge. The response in the airways induced by LPS challenge detected as an MRI signal in the lung was compared with the mucus concentration in the BAL fluid taken from the same animals killed immediately after the MRI measurement (Fig. 3). Again, the time course of the signal changes (Fig. 3A) was similar to that of the earlier studies (Fig. 2 and Table 1). The changes in mucus content of the BAL fluid followed a remarkably similar time course to that of the MRI signal (Fig. 3B). Figure 3C shows the changes in MRI signal plotted against BAL fluid mucus content for individual animals; the correlation coefficient was 0.762 (P < 0.0001, n = 36).

Histology. Figure 4 displays histological sections of lungs from animals challenged with 1 mg/kg of LPS. A substantial and sustained increase in goblet cell number was seen between 48 h and 16 days after challenge. Flocculent mucoid material was consistently detected in LPS-treated rats, rarely in controls, close to the apical surface of epithelial cells.

The number of goblet cells and the area of mucus per unit length epithelium after challenge with 1 mg/kg of LPS were quantified using labeled lectins and is presented in Fig. 5. There was a sustained and significant increase in the number of goblet cells from 48 h to 16 days after LPS challenge (Fig. 5A). Furthermore, the area of epithelial mucus staining was also significantly increased over the same time interval (Fig. 5B), consistent with the BAL fluid analysis (Fig. 3B).

DISCUSSION

Noninvasive detection and quantification of edema in the rat lung after allergen challenge in sensitized animals have been demonstrated using MRI (5). The method interferes minimally with the well-being of the animals, because neither respiratory nor electrocardiogram triggering is necessary, and the rats respire freely during data collection. The same approach has been used in the present study to detect and quantify the signal in the lung after an intratracheal challenge with LPS. At the echo time used, 2.7 ms, the signal from lung parenchyma was too weak to be detected. To observe a parenchymal signal with a reasonable signal-to-noise ratio at 4.7 T, echo times on the order of 600 μs were required (6). The absence of a lung parenchymal signal in combination with a background free of artifacts provided a high contrast-to-noise ratio for the detection of signals in response to LPS challenge.

The effects of LPS were dose dependent and remarkably long lasting; after 1 mg/kg of LPS, for example, significant signals were detected up to 16 days after challenge. Qualitatively, the signals induced by LPS appeared to have two components: the first, characterized by a diffuse appearance and stronger intensity, was particularly prominent during the first 48 h after LPS challenge; the second component, characterized by an irregular appearance and much weaker signal intensity, was also evident in the first hours after LPS challenge but predominated at later time points. The
predominance of the first component in the first 48 h after LPS challenge corresponds to the period during which neutrophil numbers, MPO activity, and protein concentration were markedly increased in the BAL fluid (Table 1). This suggests that edema resulting from generalized granulocytic (especially neutrophilic) inflammation is likely to have been the major contributor to the signal detected by MRI in the lung at the early time points.

The time course of the signal detected by MRI in the lung after LPS and that of mucus determined in the BAL fluid were highly correlated; in each case, a maximum was found 48 h postchallenge, followed by a decline. Histological analysis, on the other hand, revealed a sustained and significant increase in the number of goblet cells, as well as in the epithelial area stained for mucus from 48 h to 16 days after LPS challenge (Fig. 5). In other words, for time points later than 48 h, the volume of stored mucus remained elevated but the amount of mucus secreted decreased. Because the MRI signal elicited by LPS also decreased, the second and long-lasting component of the signal detected in the lung by MRI after LPS instillation is likely to have been due to secreted mucus. Furthermore, the fact that neither the number of goblet cells nor the mucus per unit length epithelium (Fig. 5) increased 24 h after challenge, while at the same time point significant amounts of mucus were secreted (Fig. 3), suggests that at 24 h post-LPS challenge, synthesis of mucus was keeping pace with secretion.

Fig. 4. Histological sections from animals treated with either LPS (1 mg/kg) or saline. Alcian blue-periodic acid Schiff staining for detection of goblet cells (red arrows) and flocculent mucoid material (green arrows).
is considered to primarily reflect edema, based on the good correlation between the volume of MRI signal and the protein content determined by BAL fluid analysis (5, 36).

The volume of the signal after LPS instillation was also significantly smaller (25–50%) than that observed after challenge with allergen (5). The difference could possibly be accounted for by the relative severity of the tissue eosinophilia. Thus after OA, the number of eosinophils in the BAL fluid was some three times larger than the number of eosinophils after LPS challenge (Ref. 5 and Table 1). Tissue eosinophilia results in the liberation of a multitude of proinflammatory mediators that cause smooth muscle contraction, bronchial hyperresponsiveness, vasodilation, and increased vascular leakage with the production of tissue edema (16, 23). Administration of LPS, in contrast, results in a predominantly neutrophilic infiltration orchestrated in large part by the release of TNF-α (3, 33, 37, 38). Macrophages, which are able to secrete 1,000 times more TNF-α in response to LPS than any other cell type (7, 26), were significantly elevated throughout the observation period after LPS (Table 1) and less so after OA (5). The fact that products of neutrophil (1), eosinophil (25), and macrophage (32) activation are capable of stimulating an increase in goblet cell number and/or an increase in mucus secretion would provide a plausible explanation for the greater mucus contribution to the signal after LPS than after OA.

In addition to edema and mucus, other mechanisms could potentially contribute to the signal changes described here after LPS instillation. For instance, spin-lattice (T1) and spin-spin (T2) relaxation times may reflect structural changes associated with experimental lung injury (15). Relaxation times have been measured in numerous models of lung injury, including pulmonary edema (caused, for example, by oleic acid and alloxan), bacterial and chemical inflammation, pulmonary hemorrhage, and other types of lung injury by various agents [reviewed by Shioya et al. (31)]. Cutillo et al. (11) showed an increase in T2* (20–100%) in excised, unperfused lungs removed from Sprague-Dawley rats 6 h after treatment with 10 mg/kg LPS ip. T1 was also significantly increased 6 and 9 h after endotoxin, although the changes were small (5–10%). A fivefold increase in T2* has also been observed 24 and 48 h after intratracheal challenge with LPS in regions where signals were present, whereas no significant change in T2* of parenchymal tissue was detected elsewhere in the lung (6). The good correlation between T2* assessments in edematous lung tissue and signal volumes indicates that the observed changes in T2* were primarily due to the increased water content at the sites of edematous lung tissue. A further effect that could potentially influence the signal intensity in the lung is atelectasis, which has been shown to induce changes in T2* (10). Our data do not support this concept since reduction in lung volume, a consequence of atelectasis, was observed in ~30% of saline- or LPS-challenged rats (data not shown), but signals were detected only in the lungs of LPS-treated animals.
Finally, vasoreactivity and changes in vessel volume in response to LPS could potentially affect our results. However, the fact that most of the signal in the lung after the challenge appeared in regions where, in the baseline images, no vessels had been detected indicates that changes in vessel volume contributed only marginally to the postchallenge volume of MRI lung signals reported here.

In conclusion, challenge with endotoxin induced the appearance of signals in the lungs of BN rats that differed qualitatively and quantitatively from those seen after allergen challenge in sensitized animals (5). The signals after application of LPS had two components: one of diffuse appearance and a second showing a granular pattern. The signals after application of LPS had two components: one of diffuse appearance and a second showing a granular pattern. The fact that animals that survive endotoxin challenge in actively sensitized Brown-Norway rats after single allergen exposure. 4. September 2001. 5. Harkema JR and Hotchkiss JA. In vivo effects of endotoxin on intraepithelial neutrophils in rat lung airways. Quantitative histochemistry. Am J Pathol 141: 307–317, 1992.


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