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Citation for final published version:

Evans, Rhys L., Changani, Kumar K., Hotee, Sarah, Pindoria, Kashmira, Campbell, Simon, Nials, Anthony T., Ford, William R. , Broadley, Kenneth J. and Kidd, Emma J. 2015. Pulmonary oedema measured by MRI correlates with late-phase response to allergen challenge. *Experimental Lung Research* 41 (4) , pp. 189-198.
10.3109/01902148.2014.985407

Publishers page: <https://doi.org/10.3109/01902148.2014.985407>

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Pulmonary oedema measured by MRI correlates with late-phase response to allergen challenge

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Running title: MRI measurement of lung oedema in allergic guinea-pigs

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Funding: This work was supported by a BBSRC –CASE studentship [BBS/S/N/2006/13082] in collaboration with GlaxoSmithKline, Stevenage, Hertfordshire, SG1 2NY.

ABSTRACT

Purpose: Asthma is associated with reversible airway obstruction, leucocyte infiltration, airways hyperresponsiveness (AHR) and airways remodelling. Fluid accumulation causes pulmonary oedema contributing to airways obstruction. We examined the temporal relationship between the late asthmatic response (LAR) following allergen challenge of sensitised guinea-pigs and pulmonary oedema measured by magnetic resonance imaging (MRI).

Materials and Methods: Ovalbumin (OVA) sensitised guinea-pigs received either a single OVA inhalation (acute) or nine OVA inhalations at 48 h intervals (chronic). Airways obstruction was measured as specific airways conductance (sG_{aw}) by whole body plethysmography. AHR to inhaled histamine and bronchoalveolar lavage for leucocyte counts were measured 24 h after a single or the final chronic ovalbumin challenges. MRI was performed at intervals after OVA challenge and high intensity oedemic signals quantified.

Results: Ovalbumin caused early bronchoconstriction, followed at 7 h by a LAR and at 24 h AHR and leucocyte influx. The bright intensity MRI oedema signal, peaking at 7 h, was significantly ($P < 0.05$) greater after chronic ($9.0 \pm 0.7 \times 10^3 \text{ mm}^3$) than acute OVA ($7.6 \pm 0.2 \times 10^3 \text{ mm}^3$). Dexamethasone treatment before acute OVA abolished the AHR and LAR and significantly reduced eosinophils and the bright intensity MRI oedema from 9.1 ± 1.0 to $6.4 \pm 0.3 \times 10^3 \text{ mm}^3$.

Conclusion: We show a temporal relationship between oedema and the LAR and their parallel reduction, along with eosinophils and AHR, by dexamethasone. This suggests a close causative association between pulmonary oedema and impaired airways function.

Key words: Airways hyperresponsiveness, dexamethasone, guinea-pig, magnetic resonance imaging (MRI), oedema, ovalbumin

INTRODUCTION

Asthma is a chronic inflammatory disease characterized by airway inflammation, airway hyperresponsiveness (AHR), reversible airway obstruction and airway remodelling [1]. Remodelling of the airways wall microvasculature through angiogenesis can contribute to

airways oedema [2]. Pulmonary oedema is defined as an abnormal accumulation of fluid in the extravascular compartments of the lung [3]. It can be caused by an increase in either the permeability or hydrostatic pressure within the lung blood vessels resulting in fluid escaping the microvasculature and entering the surrounding tissue [4]. The plasma leakage leading to oedema formation together with mucus hypersecretion all contribute to luminal airway narrowing and therefore impair lung function [5]. The use of magnetic resonance imaging (MRI) could prove a useful tool in evaluating oedema formation in asthmatics and in pre-clinical animal models of asthma. It could also be a valuable non-invasive methodology for determining the effects of potential asthma therapeutics on pulmonary oedema associated with asthmatic lungs.

Dexamethasone is a systemically administered corticosteroid recommended for moderate to severe exacerbations of asthma [6]. Although it is an extremely potent corticosteroid, the side-effects associated with orally administered dexamethasone limit its use [7]. Despite this, dexamethasone has been commonly used in animal models of asthma and has been effective at reducing the late asthmatic response, airway hyperresponsiveness (AHR) and cellular influx associated with asthma [8]. The use of proton MRI has been shown to provide a non-invasive measure of inflammation/oedema in the lung and this has been correlated with inflammatory cell infiltrates in bronchoalveolar lavage (BAL) samples and lung histology [9]. Typical MR images of lung are predominantly dark in appearance due to the low signal associated with lung parenchyma. Using conventional proton MRI it is also possible to detect any cell recruitment, accumulation of oedema/mucin, as well as collagen deposition within the lung due to the increase in signal that such aggregations produce. MRI of rat lungs has shown a good correlation between oedema and inflammatory cells and other inflammatory markers in BAL fluid after allergen challenge and inhibition of the oedema by budesonide [4, 10]. In the present study we apply MRI to examine whether there is a correlation between oedema and airways obstruction, together with inflammatory cell influx in allergen-challenged guinea-pigs. In particular, we also determined whether attenuation of the late asthmatic response (LAR) by the potent corticosteroid, dexamethasone, would be associated with oedema reduction.

MATERIALS and METHODS

Animals

Groups of six male Dunkin-Hartley guinea-pigs (Harlan, UK) weighing 150-175 g were sensitized and challenged with ovalbumin (OVA). The guinea-pigs either followed an acute protocol established by Smith & Broadley [11] or a chronic protocol [12]. Guinea-pigs were sensitized with bilateral intraperitoneal injections of OVA (100 µg) and Al(OH)₃ (100 mg) on days 1 and 5. On day 15, guinea-pigs following the acute protocol were challenged with inhaled OVA (0.01%, 1 h) or saline. Guinea-pigs following the chronic protocol received a further 8 exposures of OVA at 10-fold higher concentration (0.1%) or saline every 48 hours. All but the last challenge was under mepyramine cover (30 mg/kg i.p.) to prevent fatal anaphylaxis. A summary of both protocols is provided in Figure 1. All procedures involving animals were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals

Measurement of airways obstruction

Whole-body plethysmography was used to determine specific airway conductance (sG_{aw}). Guinea-pigs were placed in a restrainer with a neck clamp and a mask fitted over the snout to record airflow by means of a pneumotachograph connected to a UP1 pressure transducer. The restrainer was then placed into the plethysmograph and the endplate closed. Box pressure in the plethysmograph was measured by a UP2 pressure transducer. Respiratory flow and box pressure were converted into waveforms with Biopac system (Lectromed, UK) and specific airways conductance (sG_{aw}) determined by Acqknowledge[®] software. Readings were taken for 5 seconds which would include an average of 8 breaths. Baseline values were recorded before OVA exposure, and following the hour-long exposure, values were recorded at 0, 15, 30, 45 and 60 minutes, then hourly until 12 hours with a final reading at 24 hours. In order to capture both the early and late asthmatic bronchoconstrictor responses, the maximum bronchoconstriction occurring

between 0-6 hours and between 7-12 hours were recorded and displayed as separate histograms next to the time course plot.

Airway responsiveness

Airway responsiveness was assessed by exposing the guinea-pigs to 1 mM histamine for 20 seconds. This was done 24 hours before the single acute OVA exposure or the first of the chronic exposures and repeated 24 hours after the single or final OVA exposure. Readings of sG_{aw} were taken at baseline prior to histamine exposure and at 0, 5 and 10 minutes following the histamine exposure.

Bronchoalveolar lavage

After the final histamine exposure, the guinea-pigs were administered a lethal dose of sodium pentobarbitone (Euthatal i.p. 400 mg/kg), the trachea cannulated and the lungs removed before lavaging one lung half with saline (1 ml/100g guinea-pig) twice. Total cells were counted in the pooled samples with a Neubauer haemocytometer and differential cell counts after Cytospin centrifugation of 100 μ l of BAL fluid onto microscope slides and staining with 0.15% Leishman's solution in methanol.

Magnetic resonance imaging (MRI)

To assess *in vivo* lung inflammation MRI scanning was employed. Images were acquired using a Bruker 4.7 Tesla magnet with an 11.6 cm gradient insert and a 72 mm coil and cradle. Multislice coronal images using a gradient echo sequence was employed to acquire data from the entire pulmonary cavity. Field of view was 7.2cm, slice thickness, 1.8 mm; interslice distance, 2.4 mm using: an echo time (TE) = 2.0 ms, and a repetition time (TR) = 150 ms, 15° flip angle, spectral width = 50,000 Hz, 256 x128 matrix with 8 averages (2.5min). Before the guinea-pigs were placed in the MRI scanner they were anaesthetised with isoflurane (1-4%) and oxygen/air as the carrier for the isoflurane, this was maintained throughout the whole time they were in the machine via an actively scavenged open face mask unit. Depth of anaesthesia was measured by the animals' respiration rate which was maintained at a similar level for all animals. The guinea-pigs were placed in the supine position for imaging. As reproducing the position of the guinea-

pig for each scan was important for comparison of the images, initial ~1 min scout images were recorded to check the positioning of the axial, coronal and sagittal views. By using these scans, the final field of view for the main scan was carefully positioned using precise boundary information such as spine positioning and distances from the apex of the heart. Baseline images were captured before any challenges took place and at 15 minutes and at 4, 7 and 24 hours subsequent to the final OVA exposure. The MRI scans in figures 2 and 6 show the slices captured in one scan.

Lung volume and bright signal within the lung were quantified using Analyze 7.0 software (Mayo Clinic, KS, USA). A contour encompassing the lung was drawn and applied to each of the 7 slices of the scan (the trachea, main bronchi and heart were not included within the contour). The total volume of this lung contour was calculated. The oedematous lung area was subtracted from the main image by applying a signal intensity threshold filter (specific to each animal) to the lung contour whereby only pixels above a certain predefined intensity were included in the newly defined image. Measurement of the volume of this new image enabled the quantification of absolute bright signal (mm^3) for each scan. The mean signal intensity \pm SD of voxels associated with the oedematous lung tissue were computed by placing 6 regions of interest (ROIs) of approximately 1.2 mm radius in various areas of homogeneous intensity within the subtracted image and measuring their intensity. The signal intensity was assessed for all voxels within the previously determined lung region (within the contour). Volumetric quantitative data were calculated as the number of voxels below the calculated threshold (normal lung tissue) and above the threshold (oedematous tissue), resulting in lung and oedematous volumes (as well as mean \pm SEM intensity of oedematous voxels) [9].

Drug dosing

Dexamethasone (20 mg/kg) or vehicle (saline:DMSO 50:50) were administered by intraperitoneal bilateral injections (0.1ml) at 24 hours and 30 minutes before the OVA exposure and 6 hours after the exposure in the acute experiments.

Materials

Aluminium hydroxide, dexamethasone, dimethyl sulfoxide, histamine diphosphate, mepyramine maleate and ovalbumin were obtained from Sigma-Aldrich, Pool, UK. Sodium pentobarbitone (Euthetal) was purchased from Merial, Harlow, UK. Dexamethasone was dissolved in saline and DMSO (50:50). All other drugs and materials were dissolved in saline purchased from Baxter Healthcare, Newbury, UK.

Data and statistical analysis

Mean cell counts (\pm SEM) were recorded as cells/ml bronchoalveolar lavage fluid and mean changes in lung function (\pm SEM) were measured as the fall in sG_{aw} expressed as a percentage of the baseline value before exposure to OVA or histamine. Mean volumes of bright intensity (\pm SEM) were recorded. Comparisons between pairs of data were made by Student's paired or unpaired t-test as appropriate and between multiple groups by Analysis of Variance followed by a Bonferroni post-test. Significance was assumed when $P < 0.05$.

RESULTS

MRI scans after acute and chronic saline or OVA

Figure 2 shows sections taken from naïve, saline challenged and OVA challenged guinea-pigs 7 hours post-exposure. The level of bright intensity (white areas) is clearly greater in the two sections from the acute and chronic OVA-challenged animals. The quantified volumes of bright intensity (Table 1) show significantly greater levels after acute OVA challenge than after acute saline challenge at 4 (33.3%), 7 (29.4%) and 24 hours (44.4%)(Fig. 3a). Chronic OVA exposures led to a significantly greater intensity than chronic saline exposures at baseline (32.7%), 15 minutes (42.6%), 4 hours (50.8%), 7 hours (50.0%) and 24 hours (29.8%) following the final exposure (Fig. 3b).

Figure 3c compares acute and chronic challenges with either saline or OVA. At baseline (i.e. before the final OVA challenge) and 15 minutes and 4 hours after the final challenge, chronic OVA caused a significantly greater volume of bright intensity (82.5%) than acute

OVA challenge . However, no significant difference was seen at the 7 hour and 25 hour time points.

Airways obstruction after acute OVA challenge

An immediate bronchoconstriction characterized by a decrease in sG_{aw} followed the OVA inhalation, in the vehicle- ($-59.1\pm 5.3\%$) and dexamethasone- ($-56.8\pm 7.7\%$) treated OVA sensitized guinea-pigs. This resolved by 5 h (Fig. 4a). At 7 hours after OVA challenge, there was a second bronchoconstriction or late asthmatic response (LAR), in the vehicle-treated guinea-pigs, which was abolished by dexamethasone treatment. The mean peak sG_{aw} values for the LAR in the dexamethasone-treated group ($-5.9\pm 1.2\%$) were significantly less than after vehicle treatment ($-26.3\pm 4.8\%$).

Histamine inhalation (1 mM, 20 seconds) before OVA challenge(s) produced no bronchoconstriction as sG_{aw} did not change ($+0.1\pm 1.2\%$) (Fig. 4b). However, 24 h after the acute OVA challenge, there was a significant bronchoconstriction ($-41.4\pm 7.6\%$) immediately following the histamine challenge in vehicle-treated guinea-pigs (Fig. 4b). This was still significant 5 minutes after the histamine challenge ($-24.5\pm 4.6\%$) compared to the pre-OVA value ($-1.0\pm 2.1\%$) but resolved after 10 minutes. Dexamethasone treatment abolished this increased responsiveness to histamine with no significant difference between pre-OVA and post-OVA exposures after a histamine challenge

Bronchoalveolar lavage inflammatory cells

Acute OVA challenge of vehicle-treated animals caused significant increases in the total inflammatory cell counts and eosinophils compared with naïve animals (Fig. 5). Dexamethasone treatment significantly reduced the total number of BAL fluid cells compared to vehicle ($2.9\pm 0.2\times 10^6/\text{ml}$ compared to $6.2\pm 0.6\times 10^6/\text{ml}$ respectively) (Fig. 5). Dexamethasone also reduced eosinophil number ($2.0\pm 0.05\times 10^6/\text{ml}$ compared to $1.8\pm 0.08\times 10^6/\text{ml}$ respectively) and lymphocyte number ($0.05\pm 0.01\times 10^6/\text{ml}$ compared to $0.2\pm 0.03\times 10^6/\text{ml}$ respectively).

Effect of dexamethasone on MRI sections

Figure 6a shows a typical increase in the level of bright intensity in a single slice taken from a vehicle-treated guinea-pig at 7 hours after an acute OVA challenge. Figure 6b shows the reduced levels of bright intensity in the lungs after treatment with dexamethasone also at 7 hours after exposure to OVA. Dexamethasone significantly reduced the volume of bright intensity compared to vehicle treated guinea-pigs 7 hours after an OVA exposure ($6.4 \pm 0.3 \times 10^3 \text{ mm}^3$ compared to $9.1 \pm 1.0 \times 10^3 \text{ mm}^3$ respectively) (Fig. 6c).

DISCUSSION

We used MR imaging in guinea-pigs to demonstrate pulmonary oedema as measured by an increase in lung bright intensity following OVA challenge of OVA-sensitised animals. In the acute OVA challenged group a significant difference from the saline challenged was observed at 4 hours with the maximum levels of oedema observed at 7 hours. At 24 hours the levels of oedema were reduced suggesting some recovery. However, levels were still greater than baseline, suggesting recovery takes longer than 24 hours.

Several previous studies have also used MR imaging on animal models of asthma in rats [4, 10] and mice [9]. Lipopolysaccharide-induced pulmonary oedema has been previously measured by MRI and has shown to be inhibited following a prophylactic dose of dexamethasone [13]. Ovalbumin (OVA) sensitised and challenged Brown-Norway rats showed increased oedema 6 hours after challenge. This oedema increased to a maximum at 48 hours and then decreased at 96 hours [10]. This study also showed that oedema peaked 6 hours after repeated challenges and then steadily decreased. However, the peak level of oedema decreased after each challenge suggesting that tolerance could have occurred. Further studies in Brown-Norway rats showed a correlation between MRI high

intensity oedemic signals and BAL eosinophil number following OVA sensitisation and challenge [10]. In OVA sensitised mice an increase in oedema was observed 24 hours after the second OVA challenge [5]. However, like in the rats, the MRI high intensity oedemic signal became less intense after each challenge. By using hyperpolarized noble gases, such as ^3He and ^{129}Xe , in conjunction with MRI, it is possible to exploit the inherent low signal intensity of the lung to produce high intensity signal that reports on ventilation and function of the lung. Such techniques have been employed in mice models of lung injury [14] and this technique may complement the standard MRI techniques used here.

A somewhat unexpected finding in the present study was the presence of some oedema in the saline challenged group. This is probably due to the high fluid environment under which the guinea-pigs were exposed. The fact that maximum levels of oedema were observed at 7 hours which coincides with the peak of the LAR suggests that oedema makes a significant contribution to the LAR. But since oedema persists beyond the measurable airway function changes, it is possible that the changes in sG_{aw} are only seen when oedema reaches a threshold level.

The chronic OVA challenged group show significantly greater levels of oedema at all time points compared to saline challenge. The fact that there is an underlying level of oedema at the baseline time-point is likely to be a consequence of persistence of the oedema from the previous repeated challenges with OVA. Despite the high levels of basal oedema in the chronic OVA challenged group, the levels of oedema still increased to a maximum level 7 hours after the final challenge. In our previous studies with this chronic OVA model, we have shown that the final challenge with OVA also produces a LAR peaking at 7 hours [12]. Thus, the correlation between oedema and the LAR appears to hold true in the chronic model as well. The increase in oedema from baseline levels to 7 hours is not as pronounced as was observed in the acute OVA group suggesting that a maximal level of oedema was reached, possibly a result of the remodelling that also occurs in the airways of this chronic model [12]. When the levels of oedema were

compared between acute and chronic challenges a significantly greater baseline level was observed in the chronic OVA challenged group compared to acute OVA challenge.

Dexamethasone significantly inhibited the late asthmatic response, the AHR to inhaled histamine and the influx of inflammatory cells, confirming the anti-inflammatory nature of this corticosteroid. Dexamethasone is an orally administered corticosteroid used to treat severe asthma and its exacerbations [15] and its effectiveness against the late asthmatic inflammation was therefore expected. Eosinophils play a major role in causing the lung inflammation observed in asthma with the number of eosinophils correlating with the severity of asthma [16]. A large influx of eosinophils was observed following an OVA challenge in the vehicle-treated group compared to naïve guinea-pigs and this was significantly reduced by dexamethasone. Pre-treating guinea-pigs with dexamethasone also caused a significant reduction in the levels of oedema at 7 hours compared to vehicle treatment. Despite this, levels of oedema were still higher than naïve guinea pigs suggesting that complete eradication of pulmonary oedema is not required for late phase attenuation. This also confirms that a threshold level of oedema is required before changes in sG_{aw} can be detected.

Previous data have shown that OVA sensitization and challenge in guinea-pigs causes early and late asthmatic responses, AHR, cellular influx after acute challenge [11, 12] and additionally airway remodeling after chronic exposures [12, 17]. This study confirms that the OVA sensitization and challenge also induces pulmonary oedema which appears to correlate with the LAR. This LAR oedema can be significantly inhibited by corticosteroid treatment. Our result confirms a previous study in rats where antigen-induced LAR was associated with pulmonary oedema [18]. However, this appears to be the first study to show pulmonary oedema correlating with the LAR, both of which can be attenuated by corticosteroid treatment in OVA challenged guinea-pigs. We used OVA in the present study to produce clean allergen-induced inflammation rather than house dust mite extract which is often used now in mice but may suffer the disadvantage of having additional mechanisms of action [19]

Eosinophils, which play a key role in asthma, have been suggested to contribute towards oedema [10]. Since eosinophils have been implicated in the LAR, this could explain the correlation between oedema and the LAR. Oedema has been associated with inducing hyperresponsiveness [20] as have eosinophils [21], further corroborating the role of eosinophils in causing pulmonary oedema. Levels of eosinophils found in BAL fluid correlate with the levels of MRI high intensity oedemic signal. In rats pre-treated with budesonide a reduced level of eosinophils and MRI signal has been observed [10]. It is likely that dexamethasone has the same mechanism as budesonide, in that inhibition of eosinophil influx decreases levels of oedema, LAR and AHR. Dexamethasone treatment did not completely eliminate the level of oedema, which still peaked at 7 hours. Since the eosinophils were also not reduced to levels seen in naïve animals by dexamethasone, this suggests that low levels of activated eosinophils can still cause oedema in the lungs.

Although oedema has been shown to develop in the lungs of asthmatics [22, 23], in some animal models the levels of oedema appear to reduce after multiple challenges [5, 10]. The Tigani study implies that the reduced leakage is a result of increased vessel wall thickness following repeated allergen challenge. However, the formation of new blood vessels, angiogenesis, is associated with chronic asthma [24] suggesting that there should be more plasma leakage from an increased number of blood vessels and therefore more oedema. Indeed, in our study, the level of oedema was consistently greater after chronic OVA exposure than after acute challenge. It is likely that the decreased levels of oedema in the previous studies of Tigani *et al.* [10] and Ble *et al.* [5] are a result of allergen tolerance. Oral tolerance in sensitized animals has been shown to decrease oedema formation [25].

Conclusions

The fact that oedema was observed in the chronic OVA model, as it is in asthmatics, suggests that this is an extremely useful pre-clinical model of asthma. The use of MRI for assessing the effect of anti-inflammatory drugs could prove to be a useful tool not only in animal models but also clinically. It provides a non-invasive way of accurately assessing

the levels of oedema in the lung, a factor which appears to be highly correlated with levels of pulmonary inflammation. One issue of using MRI is that images can be distorted because of the guinea-pig's spontaneous breathing, however, taking repetitive measurements allows for a better MRI high intensity oedemic signal and therefore a clearer image and overcomes this issue.

Declaration of interest: *The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.*

Acknowledgement: *We are grateful to Dr Stephen Paisey, Positron Emission Tomography Imaging Centre, Cardiff University School of Medicine, for advice on the MRI methodology.*

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Figure Legends

FIGURE 1. Protocols for acute and chronic ovalbumin challenges of guinea-pigs and MRI scanning.

FIGURE 2. Representative MRI scans from groups of six animals obtained through the chest of a naïve guinea-pig or guinea-pigs exposed to acute or chronic inhalation challenges with ovalbumin or saline. Images were taken 7 h after the challenge and show the highest bright intensity after chronic ovalbumin exposures.

FIGURE 3. The mean (\pm SEM) volume of bright intensity in MRI scans of naïve or OVA-sensitized guinea-pigs exposed to OVA or saline. **a.** Comparison of acute saline and OVA. * significantly different from acute saline ($P < 0.05$, $n = 6$). **b.** Comparison of chronic saline and OVA. * significantly different from chronic saline ($P < 0.05$, $n = 6$). **c.** Comparison of acute and chronic saline or acute and chronic OVA. # significant difference between acute and chronic OVA challenge. The dashed line indicates the basal level of bright intensity in tissues such as the heart.

FIGURE 4. Airways function responses of OVA-sensitized guinea-pigs. **a.** Responses following a single acute OVA challenge. **b.** Responses immediately after histamine challenges 24 h before and 24 h after acute OVA challenge. Responses are plotted as the mean (\pm SEM) change in sG_{aw} expressed as a percentage of the baseline value immediately before the OVA or histamine challenge. Guinea-pigs were either treated with dexamethasone (20 mg/kg i.p.) or its vehicle (DMSO:saline, 50:50) 24 h and half an hour before and 6 h after OVA challenge. In **a**, the bar charts on the right are the mean (\pm SEM) peak changes in sG_{aw} occurring between 0-6 and 7-12 h and at 24 h after OVA challenge. In **a**, significant differences between dexamethasone and vehicle treated guinea-pigs are represented by * and ^, and in **b**, significant differences between responses to histamine before and after OVA challenge are indicated by * ($P < 0.05$, $n = 6$, Student's two-tailed t -test).

FIGURE 5. Total leucocytes, macrophages, eosinophils, lymphocytes and neutrophils in bronchoalveolar lavage fluid of naïve (non-sensitized) guinea-pigs and 24 h after an acute OVA challenge of guinea-pigs treated with dexamethasone (20 mg/kg i.p.) or its vehicle (DMSO:saline, 50:50) 24 h and half an hour before and 6 h after OVA challenge. Values are the mean (\pm SEM) numbers of cells/ml. * significantly different from naïve animals

and [^] significantly different from vehicle treated guinea-pigs by Analysis of Variance followed by Bonferroni post-test ($P < 0.05$, $n = 6$).

FIGURE 6. Representative MRI scans obtained through the chest of sensitized guinea-pigs exposed to acute inhalation challenges with ovalbumin and treated with **a.** vehicle or **b.** dexamethasone. Images were taken 7 h after the challenge and show reduced bright intensity after dexamethasone treatment. **c.** Mean volume of bright intensity (\pm SEM) in naïve and OVA sensitized acute OVA challenged guinea-pigs treated with dexamethasone (20 mg/kg i.p.) or its vehicle (DMSO:saline, 50:50) 24 h and half an hour before and 6 h after OVA challenge. The dashed line highlights the basal level of bright intensity caused by tissues such as the heart. * significantly different from vehicle treated guinea-pigs by Student's two-tailed t -test ($P < 0.05$, $n = 6$).