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Endotoxin contamination contributes to the pulmonary inflammatory and functional response to *Aspergillus fumigatus* extract inhalation in heaves horses

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Summary

Background Mould extract inhalation challenges have been used extensively in the investigation of heaves. Such challenges have induced pulmonary neutrophilic inflammation and dysfunction, consistent with, but less severe than the natural disease. However, the method of mould extract production is likely to result in endotoxin contamination.

Objective To investigate whether insufficient dose delivery was responsible for the shortfall in response to inhaled extract compared with natural disease, and whether endotoxin contamination of mould extract contributed to the pulmonary inflammation and dysfunction.

Methods We measured the response of six heaves horses following inhalation of saline (placebo) and three doses of *Aspergillus fumigatus* extract. We then compared the response of six heaves horses to *A. fumigatus* extract inhalation before and after lipopolysaccharide (LPS) depletion.

Results Inhalation challenge with 0.5, 1.6 and 5 mg of *A. fumigatus* extract resulted in a significant increase in bronchoalveolar lavage fluid (BALF) neutrophil ratio when compared with saline inhalation. Only 1.6 and 5 mg extract inhalation resulted in significant lung dysfunction compared with saline. There was no significant difference between 1.6 and 5 mg extract inhalation with respect to airway neutrophil numbers or lung function, suggesting a plateau in both measured responses. LPS depletion of 1.6 mg *A. fumigatus* extract resulted in a significant reduction in airway neutrophil numbers and increase in arterial oxygen tension. There was no significant difference between saline and the LPS-depleted *A. fumigatus* extract challenges with respect to neutrophil count and lung function. The reduction in airway neutrophil numbers was greater than would be predicted by extrapolation from previously reported soluble LPS dose–response inhalation experiments.

Conclusion This study supports a role for other inhalants, in addition to soluble components of *A. fumigatus*, in the aetiopathogenesis of heaves. Also the amplification in response to LPS when inhaled with *A. fumigatus* extract, suggests that the role of inhaled endotoxin in the pulmonary inflammation and dysfunction in naturally occurring heaves may currently be underestimated.

Keywords *Aspergillus fumigatus*, endotoxin, heaves, mould extract, organic dust

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Introduction

Heaves, a pulmonary disease of the horse characterized by airway neutrophilia and obstruction, is induced by exposure to dusty stable environments, with both the airway inflammation and dysfunction being reversible following a reduction in dust exposure [1]. Heaves is therefore similar to human occupational asthma in workers chronically exposed to organic dust [2]. Although previous mould extract inhalation studies have implicated a hypersensitivity to inhaled moulds in disease pathogenesis [3–5], little attention has been given to the additional role of other inhaled dust components. Experimental mould extract inhalation induces pulmonary neutrophilic inflammation and dysfunction, consistent with, but less severe than the natural disease [4]. Despite one suggested reason for this relatively less severe response being insufficient dose delivery, no dose–response inhalation challenges have yet been reported. Other reasons include the involvement of other dust components in the overall pulmonary response and recent work has concentrated on the potential role of endotoxin for two main reasons. First, dust collected from stables contains relatively high quantities of endotoxin [6–9], and second, lipopolysaccharide (LPS) inhalation challenges in horses, results in obstructive lung dysfunction and neutrophilic airway inflammation, both characteristic features of naturally occurring heaves [10]. Despite proposed similarities to human asthma, eosinophil airway infiltration is not as feature of heaves. However, even in asthma, it is possible that endotoxin contamination of allergen can alter the predominant cell population

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from eosinophils to neutrophils following inhalation challenge [11, 12]. Indeed, endotoxin contamination of inhaled allergens is one theory for the neutrophilic lung infiltration reported with sudden-onset fatal asthma [13]. It is therefore possible that the high concentration of endotoxin to which horses are exposed is responsible for the neutrophilic inflammation characteristic of heaves. This paper describes for the first time a series of dose–response inhalation experiments in six heaves horses, using *A. fumigatus* extract. Additionally, following detection of LPS contamination of the extract, the response to inhalation challenge with LPS-depleted extract was measured and compared with the response prior to depletion.

Materials and methods

Horses

Six horses (three geldings, three mares; median age 17 years, range 8–28 years; median weight 434 kg, range 323–594 kg) with a history and clinical diagnosis of heaves were used for this study. The disease status of all subjects was confirmed by previous mouldy hay/straw challenges of varying durations. These challenges induced bronchoalveolar lavage fluid (BALF) neutrophilia ($> 20\%$), increased volume of tracheal secretions, a reduced PaO_2, an increase in maximum transpulmonary pressure (2.41 ± 1.04 kPa; mean $\pm$ SD), isovolumetric lung resistance (0.24 ± 0.09 kPa/l/s) and work of breathing (92.2 ± 42.6 J/min), and a decrease in dynamic compliance (7.48 ± 5.8 L/kPa) in all heaves horses. All of the above clinical and laboratory abnormalities reverted to normal when the heaves horses were moved to a low-dust environment. All horses were kept in a low-dust environment for a minimum of 6 months prior to the study and throughout the duration of this study. The study was approved by the Home Office, and conducted under a Home Office project licence.

Inhalation challenges

Nebulized inhalation challenges were performed as previously described [10], using 0.5, 1.6 and 5 mg *A. fumigatus* extract (AFE) and endotoxin-depleted AFE (AFE-LPS). To facilitate nebulization, horses were sedated with 20 g/kg romifidine (Sedivet, Boehringer Ingelheim Ltd, Bracknell, Berkshire, UK) and 10 g/kg butorphanol (Torbugesic, Fort Dodge Animal Health, Hedge End, Southampton, UK), intravenously. The aerosol was generated using a compressor (Parimaster, PARI Medical Ltd, West Byfleet, Surrey, UK), with a calibrated output of 7 L/min, connected to a nebulizer cup (Sidestream, Medic-Aid Ltd, Bognor Regis, West Sussex, UK), the manufacturers of which state that 80% of aerosol is in the respirable range (< 5 m). The nebulizer cup contained 2 mL of challenge solution. The aerosol passed via a 'T piece' system into an airtight facemask, with inspiratory and expiratory valves to minimize aerosol loss. One microlitre of solution was delivered to the facemask for each challenge. AFE was prepared from both cellular (somatic) and extracellular (culture filtrate) components of *A. fumigatus* culture (kindly donated by Dr John Edwards, MRC Immunobiology Laboratory, Sully Hospital, Penarth, Wales, UK). The strain of *A. fumigatus* used in the extract was I355, previously shown to produce a high quantity and quality of antigens following double dialysis preparation [14].

The three doses of AFE were prepared from a stock solution of 10 mg/mL and diluted in physiologic saline (Vetivex, 0.9% sodium chloride 0.9% w/v, Ivex Pharmaceuticals, Larne, UK) immediately prior to use.

The responses to the AFE challenges were compared with those to saline inhalation (placebo control) and a conventional mouldy hay/straw challenge (positive control; as previously described [10]).

The endotoxin content of the AFE before and after LPS depletion were determined using an endotoxin-specific assay (Endospecy, Seikagaku Co, Tokyo, Japan) as described by Thorn [15]. To achieve endotoxin depletion, AFE (10 mg/mL) was mixed with polymixin-coated beads in 50% glycerol (Polymixin B-agarose, Sigma-Aldrich Co. Ltd, Dorset, UK) for 30 min. The resulting mixture was centrifuged (1600 ***g***; 15 min) to pellet the beads, and the LPS-depleted supernatant (AFE-LPS) was decanted and stored at $-80\,^{\circ}$C. As the glycerol was not separated from the AFE during centrifugation, the resultant AFE-LPS was diluted with glycerol, resulting in a concentration of *A. fumigatus* of 5 mg/mL in AFE-LPS. This solution was diluted in saline to achieve a concentration of 1.6 mg/mL *A. fumigatus*, which was used in the inhalation challenges. For all inhalation challenges, a 1-mL volume of challenge solution was delivered to the facemask.

To minimize potential carry-over effects of a preceding challenge on subsequent challenges, inhalation challenges were conducted a minimum of 14 days apart, and all horses were shown to have normal clinical and lung function findings immediately prior to each inhalation challenge. Additionally, all horses were shown to have less than 5% neutrophils in BALF collected from the left diaphragmatic lobe a minimum of 5 days prior to each inhalation challenge with AFE or AFE-LPS. The order of challenges was the same for each subject, namely saline, 5 mg AFE, 0.5 mg AFE, 1.6 mg AFE and, finally, AFE-LPS.

Hay/straw challenges

The hay/straw challenges were conducted a minimum of 12 weeks prior to the AFE challenges. The same six heaves horses were housed for 5 h in two small (3.7×3.7 m), poorly ventilated stables with the doors and air vents closed, bedded on deep litter straw and fed a mixture of good quality and mouldy hay. This hay/straw challenge has been shown to induce heaves only in susceptible horses [4]. The endotoxin exposure in the horses' breathing zone was measured during the 5-h hay/straw challenge, using the methods previously described [10].

Assessment of response to challenges

The method and timing of assessment of response to each challenge is summarized in Fig. 1. The response to nebulized inhalation challenges were assessed using clinical scoring, arterial blood gas analyses (AVL Opti CCA, AVL Medical Instruments UK Ltd, Stone, Staffs, UK) and BALF cytology as previously described [10, 16, 17] as well as lung mechanics, airway reactivity. BALF was collected from the right accessory lobe. All lung function measurements were performed on standing unsedated horses. Respiratory flow was measured using a heated pneumotachograph (A. Fleish No.4, Bilthoven, the Netherlands), mounted on an airtight facemask and

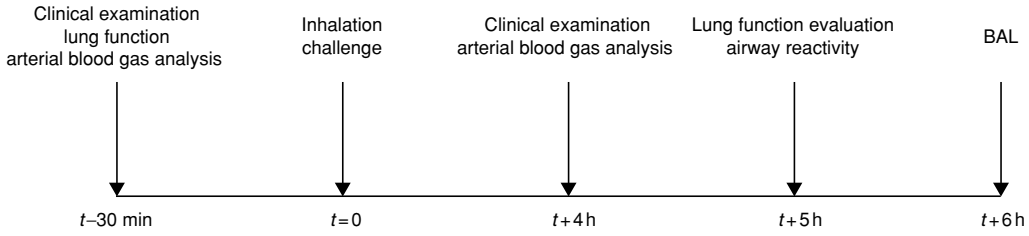

Fig. 1. Study design. For the 5 h duration hay/straw challenges, the horses entered the stable at $t = 0$. BAL, bronchoalveolar lavage.

transpulmonary pressure was measured using a balloon catheter positioned in the distal thoracic oesophagus. Both instruments were connected to pressure transducers, the output of which were conditioned, amplified as necessary and converted from analogue to digital form, using appropriate hardware (National Instruments Co., Austin, TX, USA). Custom-designed computer software (Labview, National Instruments Co., Austin, TX, USA) was used to facilitate integration of the flow signal to yield tidal volume. From each measurement period, a minimum of six total representative breaths, devoid of artefacts, were selected for further analysis. For each breath, custom designed software (Labview, National Instruments) was used to derive 21 separate lung function indices from flow, transpulmonary pressure and tidal volume.

To ensure safety and subject co-operation, horses were sedated, as described above, for the measurement of airway reactivity. Baseline lung function measurements were made following aerosol challenge with saline. Subjects then received aerosol challenge with doubling concentrations of methacholine chloride (Sigma Aldrich) dissolved in saline, with lung function measurements made following each challenge. Airway reactivity was defined as the concentration of inhaled methacholine chloride solution required to reduce the dynamic compliance to 70% of the baseline value.

Statistical analyses

The effects of each inhalation challenge were determined by performing within-group analyses. Where pre-challenge measurements were made at $t = -30$ min (arterial blood gas analyses and lung mechanics) the post-challenge values were expressed as percentage of baseline value, except for clinical scores, where actual values were used. As the vehicle for all nebulized inhalation challenges was saline, the effect of these challenges was assessed by pairing and subtracting post-challenge and post-saline data. Where no pre-challenge data was collected, comparisons were made with saline challenge data.

A Friedman test was performed on sets of paired data, and when significant, a Wilcoxon rank sum test was performed on paired data. Significance was assumed if $P < 0.05$. Results are expressed as median and range. Changes from baseline and comparison of medians between challenges are expressed as increase/decrease or difference in median values, with 95% confidence interval for the difference in median, calculated for non-parametric data as described by Campbell and Gardner [18]. As an indication of agreement for the 1.6 and 5 mg AFE challenges, the differences in paired values were plotted against their mean, as described by Bland and Altman [19], for BALF neutrophil counts. Good agreement was assumed if the calculated differences in paired values fell within 2SD of the mean of the differences [20].

Results

Endotoxin exposure during hay/straw, 1.6 mg AFE and AFE-LPS challenges

The median endotoxin concentration of the respirable dust collected from the horses' breathing zones during the hay/straw challenges was 8.7 EU/m^3 (range 4.3–10.3 EU/m^3). The endotoxin concentration of the 10 mg/mL stock solution of AFE was 31 490 EU/mL. The endotoxin content of AFE-LPS (5 mg/mL *A. fumigatus*) was 5048 EU/mL. As both the AFE and AFE-LPS were diluted in physiologic saline to result in a final challenge concentration equivalent to 1.6 mg *A. fumigatus*, polymixin treatment reduced the endotoxin concentration delivered to the facemask from 5038 EU [31 490 EU/mL $\times$ (1.6/10)] to 1615 EU [5048 EU/mL $\times$ (1.6/5), i.e. a reduction of 3423 EU, equating to a 68% reduction in endotoxin activity.

Dose–response to AFE inhalation challenge

The BALF total and differential cell counts are summarized in Table 1. Compared with saline inhalation, both 1.6 mg and 5 mg AFE inhalation resulted in a significant ($P < 0.05$) increase in BALF neutrophil count (1.6 mg, median increase in median 0.84×10^5/mL, 95% CI 0.65–2.02; 5.0 mg, median increase in median 0.95×10^5/mL, 95% CI 0.31–1.76) and ratio, at 6 h (Table 1; Fig. 2). Although inhalation of 0.5 mg AFE did not result in a significant increase in BALF neutrophil count, it did induce a slight, yet significant ($P < 0.05$) increase in BALF neutrophil ratio (Table 1). When compared with 0.5 mg AFE inhalation, both 1.6 and 5 mg AFE inhalation resulted in a significant ($P < 0.05$) greater BALF neutrophil count (1.6 mg, median difference in median 0.78×10^5/mL, 95% CI 0.37–1.89; 5.0 mg, median difference in median 0.85×10^5/mL, 95% CI 0.29–1.35) and ratio, at 6 h (Table 1; Fig. 2). A 5 h hay/straw challenge in the same six horses induced a significantly ($P < 0.05$) greater BALF neutrophil count and ratio than 0.5 mg AFE, and a greater BALF neutrophil count and ratio than 1.6 and 5 mg, which approached significance ($P = 0.059$; Fig. 2). There was no significant difference in the neutrophil count or ratio between the 1.6 and 5 mg AFE challenges. In addition, as all of the six calculated differences in total BALF neutrophil number values for the 1.6 and 5 mg AFE challenges fell within 2SD of the mean of the differences, and as the mean of the differences approximated zero, agreement was considered good [19]. Both of these findings were suggestive of a plateau in the neutrophilic response (Fig. 2).

For comparative purposes, raw data for expiratory resistance at 25%, 50% and 75% of tidal volume (RLE25%, RLE50% and RLE25%, respectively) before and after each challenge are presented in Table 2. Following correction for any effects of saline

Table 1. Total (TCC) and differential BALF cell counts ($\times 10^5$/mL) and BALF neutrophil ratio (%) in heaves ($n = 6$) horses at 6 h following inhalation challenge with saline, 0.5, 1.6 and 5.0 mg AFE and AFE-LPS, and hay/straw challenge (H/S) – median and range

	Neutrophils		TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
	Ratio	Count						
Saline	2.2 (0.6–4.5)	0.07 (0.03–0.20)	4.50 (3.20–5.60)	2.22 (1.54–3.33)	1.77 (1.24–2.96)	0.14 (0.09–0.19)	0.01 (0.01–0.11)	0.00 (0.00–0.01)
0.5 mg AFE	6.8* (2.1–16.2)	0.18 (0.07–0.48)	3.45 (1.50–5.10)	1.38 (0.36–2.01)	1.72 (0.85–2.56)	0.04 (0.03–0.11)	0.00 (0.00–0.02)	0.00 (0.00–0.02)
1.6 mg AFE	26.7* (11.3–53.9)	0.94*† (0.68–2.10)	3.85 (2.70–9.40)	1.21 (0.56–6.04)	1.57 (0.72–2.78)	0.05* (0.04–0.14)	0.00* (0.00–0.02)	0.00 (0.00–0.01)
5.0 mg AFE	24.5* (7.9–44.5)	1.08*† (0.36–1.83)	4.45 (2.00–6.60)	1.86 (0.74–2.38)	1.62 (0.33–2.69)	0.05 (0.02–0.14)	0.00* (0.00–0.01)	0.01* (0.00–0.02)
1.6 mg AFE-LPS	12.8*‡ (5.0–47.5)	0.44‡ (0.16–1.90)	3.85 (2.70–5.60)	1.30 (1.09–2.37)	1.58 (0.76–2.61)	0.09‡ (0.05–0.15)	0.01 (0.00–0.06)	0.00 (0.00–0.01)
H/S	36.0*† (21.0–60.7)	1.97*† (0.74–9.83)	6.35 (2.80–16.20)	1.87 (0.60–4.26)	1.73 (0.90–3.16)	0.10 (0.08–0.12)	0.01 (0.00–0.05)	0.00 (0.00–0.02)

*Significantly different from saline ($P < 0.05$); †significantly different from 0.5 mg AFE ($P < 0.05$); ‡significantly different from 1.6 mg AFE ($P < 0.05$).

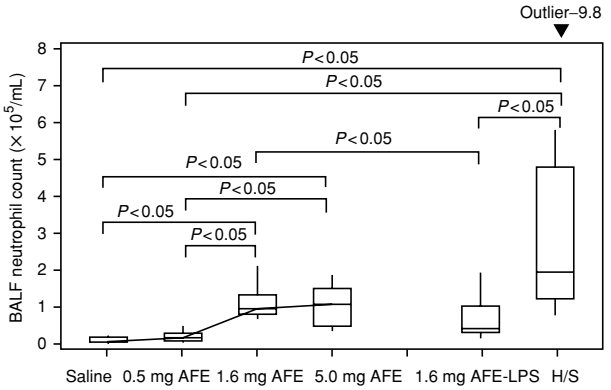

Fig. 2. BALF neutrophil counts ($\times 10^5$/mL) in heaves ($n = 6$) horses at 6 h following inhalation challenge with saline, 0.5, 1.6 and 5.0 mg AFE, AFE-LPS and mouldy hay/straw challenge (H/S), respectively.

inhalation, a dose-dependant alteration in lung function was detected following AFE challenge, with both 1.6 mg and 5 mg, but not 0.5 mg AFE inhalation, resulting in a significant ($P < 0.05$) increase in RLE25% (1.6 mg, median increase in median 94%, 95% CI 14–678; 5 mg, median increase in median 114%, 95% CI 12–578) at 5 h. The 5 h hay/straw challenge did not result in any significant alteration in lung function when compared with baseline values. There was no significant difference between the 1.6 and the 5 mg AFE challenges with respect to RLE25%. In addition, as all of the six calculated differences in RLE25% values for the 1.6 and 5 mg AFE challenges fell within 2SD of the mean of the differences and the mean of the differences approximated zero, agreement was considered good [19]. Both of these findings were also suggestive of a plateau in the lung function response. None of the AFE doses induced a significant alteration in clinical score or blood gas profiles (Table 3) at 4 h, or airway reactivity at 5 h (Table 4).

Response to inhalation challenge with AFE and AFE-LPS

The alteration in BALF cytology following inhalation challenge with 1.6 mg AFE is described above. Following LPS depletion, inhalation challenge with AFE-LPS resulted in a significant ($P < 0.05$) reduction in BALF neutrophil count (median decrease in median 0.43×10^5/mL, 95% CI 0.10–0.65) and ratio at 6 h when compared with AFE challenge (Table 1; Fig. 2). In addition, the BALF neutrophil count following AFE-LPS challenge was not significantly different from saline challenge (Table 1; Fig. 2).

LPS depletion also resulted in a significant ($P < 0.05$) increase in the BALF mast cell count (median increase in median 0.03×10^5/mL, 0.01–0.06) at 6 h, compared with 1.6 mg AFE challenge (Table 1). The BALF mast cell and basiphiloid cell numbers 6 h following AFE-LPS challenge were not significantly different than the saline challenge. Neither the AFE nor AFE-LPS challenges resulted in a significant alteration in total BALF cell numbers or absolute lymphocyte, macrophage, eosinophil or epithelial cell numbers.

The significant ($P < 0.05$) increase in RLE25% at 5 h following 1.6 mg AFE challenge did not occur following AFE-LPS challenge. In addition, when AFE and AFE-LPS challenges were compared with respect to alteration in blood gas indices, AFE challenge induced a significantly ($P < 0.05$) greater decrease in median arterial oxygen tension at 4 h than

Table 2. RLE25%, RLE50% and RLE75% (kPa/l/s) in heaves ($n = 6$) horses prior to (B/L) and at 5 h following inhalation challenge with saline, 0.5, 1.6 and 5.0 mg AFE and AFE-LPS, and hay/straw challenge (H/S) – median and range

	Time point	Saline	0.5 mg AFE	1.6 mg AFE	5.0 mg AFE	1.6 mg AFE-LPS	H/S
RLE25%	B/L	0.05 (0.04–0.09)	0.04 (0.01–0.07)	0.03 (0.01–0.06)	0.03 (0.01–0.06)	0.06 (0.01–0.08)	0.03 (0.02–0.17)
	5 h	0.03 (0.03–0.06)	0.03 (0.02–0.07)	0.05 (0.03–0.13)	0.06 (0.02–0.07)	0.07 (0.03–0.10)	0.05 (0.01–0.11)
RLE50%	B/L	0.06 (0.05–0.08)	0.05 (0.03–0.07)	0.03 (0.02–0.06)	0.03 (0.01–0.06)	0.06 (0.06–0.10)	0.05 (0.03–0.13)
	5 h	0.04 (0.01–0.06)	0.04 (0.04–0.07)	0.07 (0.03–0.12)	0.06 (0.01–0.08)	0.07 (0.05–0.11)	0.06 (0.01–0.13)
RLE75%	B/L	0.08 (0.06–0.08)	0.06 (0.05–0.08)	0.06 (0.05–0.08)	0.05 (0.03–0.06)	0.08 (0.06–0.17)	0.07 (0.06–0.19)
	5 h	0.05 (0.04–0.07)	0.05 (0.05–0.08)	0.09 (0.05–0.13)	0.06 (0.03–0.08)	0.11 (0.08–0.15)	0.07 (0.05–0.14)

Table 3. Arterial blood pH, pO_2 (mmHg) and pCO_2 (mmHg) in heaves ($n = 6$) horses prior to (B/L) and at 4 h following inhalation challenge with saline, 0.5, 1.6 and 5.0 mg AFE and AFE-LPS, and hay/straw challenge (H/S) – median and range

	Time point	Saline	0.5 mg AFE	1.6 mg AFE	5.0 mg AFE	1.6 mg AFE-LPS	H/S
pH	B/L	7.38 (7.35–7.41)	7.39 (7.35–7.41)	7.38 (7.36–7.41)	7.37 (7.33–7.39)	7.39 (7.37–7.41)	7.39 (7.37–7.45)
	4 h	7.38 (7.35–7.43)	7.40 (7.40–7.42)	7.38 (7.37–7.43)	7.39 (7.35–7.40)	7.39 (7.36–7.43)	7.42 (7.39–7.46)
pCO2	B/L	45.5 (42.0–53.0)	48.3 (45.6–50.9)	48.6 (41.6–52.4)	48.2 (43.9–52.8)	49.7 (43.3–54.9)	42 (35–50)
	4 h	44.0 (37.0–46.0)	47.6 (46.0–53.8)	48.3 (43.0–53.1)	48.1 (46.5–54.4)	48.6 (43.8–51.8)	43 (36–47)
pO2	B/L	99.5 (94.0–116.0)	102.2 (94.3–113.6)	113.5 (102.7–116.2)	105.4 (87.8–109.9)	99.3 (89.3–104.4)	103 (94–114)
	4 h	98.0 (83.0–109.0)	99.0 (93.0–112.1)	102.9 (99.3–110.3)	101.9 (87.8–108.7)	102.8 (100.9–111.0)	94 (86–107)

Table 4. PCCdyn70 values (mg/mL) in heaves ($n = 6$) horses at 5 h following inhalation challenge with saline, 0.5, 1.6 and 5.0 mg AFE and AFE-LPS, and hay/straw challenge (H/S) – median and range

Challenge	Saline	0.5 mg AFE	1.6 mg AFE	5.0 mg AFE	1.6 mg AFE-LPS	H/S
PCCdyn70	6.02 (3.06–10.53)	7.30 (2.45–24.57)	5.15 (0.72–9.57)	5.60 (4.18–11.02)	7.89 (4.42–11.24)	5.54 (2.15–14.04)

AFE-LPS (median difference in median – 11%, 95% CI 4–27; Table 3). Neither AFE nor AFE-LPS challenge resulted in an alteration in clinical score at 4 h, or airway reactivity at 5 h (Table 4).

Discussion

This paper reports for the first time the results of a series of dose–response inhalation experiments in heaves horses using soluble mould extract. In agreement with other studies, mould extract inhalation resulted in both pulmonary inflammation and dysfunction [4, 5]. Both the inflammatory and functional responses were dose-dependent.

The response threshold for lung dysfunction (0.5–1.6 mg AFE) was higher than the response thresholds for inflammation (< 0.05 mg AFE). Although this indicated that markers of inflammation were more sensitive indices of the effects of inhaled AFE than lung dysfunction, the two separate response thresholds were relatively similar. This is in contrast to the response thresholds following LPS dose–response inhalation studies in the same horses, whereby significant airway inflammation was detected at an exposure 100-fold lower than that which induced significant lung dysfunction [10]. Despite the significant increase in pulmonary resistance measured following inhalation challenge with 1.6 and 5.0 mg AFE, it should be noted that this dysfunction was not of sufficient severity to be clinically detectable. This is likely to reflect the relative insensitivity of pulmonary mechanics testing in the horse when measured during tidal breathing. Although there appeared to be a trend for increased airway reactivity with increasing doses of AFE, no significant alteration in airway reactivity was detected. Although this finding may result from insufficient AFE exposure, it may also reflect an attenuation of the methacholine-induced bronchconstriction by the bronchodilatory effects of the α2-agonist drug [21] used to sedate horses for this procedure.

In retrospect, randomization of the challenge doses would have been more appropriate to support a dose-dependent response to increasing AFE exposures. However, it is unlikely that a carry-over effect of the high-dose challenge resulted in an exaggerated response to the middle dose. First, all horses received a 0.5-mg AFE challenge following the 5-mg dose, yet only a minor response to this low dose was detected. Second, a lack of carry-over effects was supported by the lack of any significant BALF neutrophilia prior to each challenge and the failure to detect any significant differences between the baseline RLE25% values and arterial blood gases and pH measurements.

Although the agreement between the inflammatory and functional responses to the 1.6 and 5 mg exposures was suggestive of a plateau in response, higher challenge exposures would be

required to further investigate this phenomenon. Similar plateaus in response have been reported previously in relation to both skin reactivity in children with allergic eczema, following atopy patch testing [22], and pulmonary inflammation in a guinea pig model of asthma following dust mite extract inhalation [23]. As previous studies have proposed the role of a type 1 hypersensitivity response in the pathogenesis of heaves [3–5], with a suggested role for pulmonary mast cells [24], it is feasible that a plateau in indices relating to airway constriction (e.g. pulmonary resistance) may reflect a state of 'allergen saturation'. Additionally, if mast cell degranulation contributes to neutrophil infiltration as has been suggested via tryptase-mediated upregulation of IL-8 [25], the plateau in airway neutrophil numbers may also be attributed to such a phenomenon.

Although only the 0.5 mg AFE exposure resulted in a significantly lower BALF neutrophil count than the hay/straw challenge performed in the same horses, the difference between the neutrophilic response to hay/straw challenge and both the 1.6 and 5 mg AFE exposures approached significance, with the failure to achieve statistical significance in both cases being attributed to the same horse. These findings are consistent with those of previous mould inhalation studies whereby the severity of pulmonary inflammation and dysfunction associated with the natural disease was not reproduced [4]. Although many explanations for this shortfall in response have been proposed [4], the results of the current study are supportive of the role of inhalants other than those present within AFE in the natural disease. This is perhaps not surprising considering the myriad of inhalants present within stable dust, many of which have proinflammatory properties [26–28], including endotoxin.

The finding of endotoxin contamination of AFE offered a suitable model to further investigate the role of endotoxin in the aetiopathogenesis of heaves and the successful depletion of LPS from dust extracts using polymixin has been previously described [29, 30]. The reduction in the neutrophilic response and in lung dysfunction following challenge with LPS-depleted AFE was greater than was predicted upon extrapolation from previous LPS dose–response inhalation experiments [10]. Polymixin treatment of the AFE equated to a reduction in delivery to the facemask equivalent to only 3423 EU. Previous inhalation challenges in the same six horses, with 48 077 EU soluble LPS resulted in an increase in BALF neutrophil numbers (median increase in median 0.20×10^5/mL, 95% CI 0.06–0.48), compared with saline at 6 h [10]. Therefore, the difference in the median BALF neutrophil count when the depleted (equivalent to 1615 EU) and non-depleted (equivalent to 5038 EU) AFE challenges were compared was greater than that induced following 48 077 EU LPS inhalation. This finding suggests that the LPS content of the extract contributed to the pulmonary inflammatory response following inhalation, to a greater degree than would be predicted if the contribution was solely additive to that of AFE.

Despite the fact that the AFE-LPS challenge consistently resulted in a BALF neutrophil count not significantly different from saline challenge, it is unlikely that the response to AFE could entirely be attributed to the activity of LPS. Previous LPS dose–response experiments have demonstrated that a substantially higher dose of LPS than that present within both the 1.6 and 5 mg AFE (5038 and 15 744 EU, respectively) is required to induce an equivalent airway neutrophilia and lung dysfunction [10].

It could be argued that the endotoxin activity present within the AFE, and thus that removed during polymixin treatment, resulted from a different LPS type compared with the Salmonella mutant used in the previous LPS challenge experiments [10], thus precluding comparisons between the different studies. However, this LPS, an Ra chemotype (i.e. complete core oligosaccharide plus lipid A) represents a structure shared by many of the Enterobacteriaceae, including all *Escherichia coli* and Salmonella species, and this common structure is responsible for a major part of the biological activity of LPS (I. R. Poxton, personal communication). It would have been interesting to replace the depleted LPS using the Salmonella R60 mutant. Re-establishment of the neutrophilic response to a degree similar to that following challenge with non-depleted AFE would have supported the theory that the depleted LPS and the Salmonella R60 mutant had similar biological activities. This would also have provided confirmation that the reduction in response resulted entirely from LPS depletion, and was not due to any alteration in the activity of other agents present within AFE. This would also have confirmed that the glycerol contamination within the AFE-LPS challenge did not affect the response; however, the authors experience with other inhalation challenges, in which LPS had been added back to depleted suspensions containing glycerol residues, fails to support any role for glycerol in the attenuation of the inflammatory response.

In agreement with the current study, the phenomenon of disease severity being related to endotoxin exposure, in addition to allergen exposure, has been documented in human asthma [31–34]. Similarly, co-exposure of a murine model of asthma to LPS and allergen results in a greater degree of airway neutrophilia, when compared with allergen challenge alone [35]. It is possible that the presence of LPS contamination in the current AFE model resulted in a magnification in the response to mould allergens present within the extract, consistent with previous studies which have demonstrated an augmentation of the immunoglobulin responses to allergen by LPS [36–38]. Alternatively, the response to inhaled LPS may have been magnified by the co-presence of allergen, perhaps via an increase in the concentration of LPS-binding protein and soluble CD14 receptors in the bronchoalveolar compartment after allergen challenge, as has been demonstrated in human asthma [39, 40].

Interestingly, an alteration in the type of cellular response to inhaled allergen has also been demonstrated in man following endotoxin contamination of allergen, whereby neutrophils instead of eosinophils were the predominant cell type detected within the airways [11, 12]. It is possible therefore that endotoxin contamination of mould extracts used in previous investigations of heaves contributed significantly to the reported neutrophil influx in the airways [3, 4]. Although reduction in the LPS content of AFE in the current study did not alter the type of inflammatory cell recruited to the airways from neutrophils to eosinophils, complete LPS depletion was not achieved. Consequently, the residual level of endotoxin contamination in the AFE-LPS challenge, albeit reduced, may have contributed to the neutrophilic influx.

In conclusion, this study supports the role of other inhalants in addition to soluble mould allergens in the aetiopathogenesis of heaves. Considering the wide variety of inhalants to which stabled horses are exposed, the clinical features of this disease may reflect a hypersensitivity to inhaled mould allergens, which

results in a magnification in the host response to other proinflammatory components of stable dust, for example endotoxin.

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