

Kinetics of pulmonary neutrophil recruitment and clearance in a natural and spontaneously resolving model of airway inflammation

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Summary

Background Neutrophil apoptosis and phagocytic clearance have been proposed as key determinants affecting the resolution of airway inflammation.

Objective To determine the kinetics of neutrophil priming, recruitment, activation and subsequent clearance in a naturally occurring equine disease model of neutrophilic pulmonary inflammation.

Methods and Results A 5 h mouldy hay/straw challenge in hypersensitive horses induced transient pulmonary dysfunction lasting 4 days. At 24 h circulating neutrophils were primed and displayed delayed rates of spontaneous apoptosis *in vitro*. Neutrophil numbers in the airspaces peaked at 5 h and then fell abruptly, returning to pre-challenge levels by 4 days. Airspace neutrophils demonstrated increased respiratory burst activity compared with circulating cells and equine neutrophil elastase 2A concentrations increased in parallel with neutrophil numbers indicating *in vivo* priming and degranulation. The number of apoptotic neutrophils and proportion of alveolar macrophages containing phagocytosed apoptotic neutrophils increased significantly at 24 h and 4 days post-challenge corresponding to the period of most rapid neutrophil clearance.

Conclusion This is the first demonstration of spontaneous neutrophil apoptosis and phagocytic removal in a natural disease model of airway inflammation and provides critical kinetic data to support the hypothesis that this clearance pathway plays a central role in the resolution of neutrophilic inflammation.

Keywords airway inflammation, apoptosis, horse, neutrophil, priming

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Introduction

The neutrophil plays a vital role in antibacterial host defense. However, because of their potential to release large amounts of histotoxic and other pro-inflammatory agents, this cell also has the capacity to produce significant tissue injury. Hence inappropriate or excessive activation of neutrophils *in vivo* has been implicated in the pathogenesis of a wide variety of inflammatory diseases. It is not surprising therefore that the delicate gas-exchange units and conducting airways of the lung are particularly vulnerable to such damage.

It is apparent however that the presence of neutrophils alone does not necessarily equate with tissue injury and that even very intense neutrophil accumulation as seen in human streptococcal lobar pneumonia can resolve without causing major structural damage. A process that may underlie such differences in neutrophil behaviour is the ability of priming agents such as lipopolysaccharide (LPS) to up-regulate the

response of neutrophils to secretagogue stimuli. Indeed, priming has been shown to be a pre-requisite for secretagogue-induced neutrophil-mediated endothelial injury both *in vitro* [1] and in experimental lung injury *in vivo* [2]. Moreover, primed neutrophils are more adhesive and less deformable than unprimed cells and these events greatly amplify the accumulation of neutrophils in the pulmonary microcirculation.

In contrast, it is now well recognized that neutrophils are programmed to undergo constitutive apoptosis. This process locks the cell into a non-secretory mode and initiates rapid engulfment and removal by inflammatory macrophages. The potential pathophysiological importance of this event has been suggested by studies demonstrating a wave of granulocyte apoptosis occurring during the resolution phase of a number of experimental models of inflammation [3, 4] and in patients with acute asthma receiving corticosteroid therapy [5]. The observation that many pro-inflammatory agents, most notably C5a, IL-8, granulocyte macrophage-colony stimulating factor (GM-CSF) and LPS, cause a profound inhibition of neutrophil apoptosis as well as cell priming, suggests that these processes may be linked. Although such studies indicate that apoptosis is the primary mode of granulocyte cell death *in vitro* and that this process occurs *in vivo*, the experimental systems used to date have not

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determined the role of neutrophil apoptosis in the resolution of inflammation in a natural disease setting. Likewise, these studies have not addressed the relationship between neutrophil priming (both in circulating and post-migratory cells) and the onset of apoptosis at the site of inflammation.

To address these questions we established an equine model of natural antigen-induced neutrophilic bronchiolitis. Exposure of susceptible animals to mouldy hay and straw challenge causes the clinical condition of heaves that is characterized by the rapid onset of airway inflammation and hypersecretion resulting in airway obstruction, bronchial hyper-responsiveness and the disruption of pulmonary gas exchange. It is believed to result from the inhalation of organic dusts containing multiple allergens including the spores of *Aspergillus fumigatus*, *Faenia rectivirgula* and thermophilic actinomycetes [6, 7]. The clinical importance of this condition is reflected by data indicating that this is the most commonly recognized chronic pulmonary disease in the horse [8] and in some Northern European countries is the principle cause of premature equine retirement or death [9, 10]. Of particular relevance to the current study, analysis of the cellular and inflammatory response in the airways of these animals reveals a rapid onset of intense neutrophilic airspace inflammation, which occurs in the absence of any change in eosinophil numbers [6, 7]. Likewise the condition improves rapidly when the inciting challenge is removed and horses in remission are clinically indistinguishable from healthy non-susceptible animals. This natural disease model also permits serial sampling of the lower airways and hence allows a detailed kinetic analysis of the onset and recovery of the lung inflammatory response and the ability to correlate such events with changes in peripheral blood neutrophil numbers and function.

Materials and Methods

Experimental animals and challenge protocols

All studies were performed with ethical approval granted under UK Home Office Licence. Six mixed breed horses, median age 17.5 years (range 12–30), median weight 518 kg (range 454–580) with a history of heaves were housed in a controlled minimal dust environment in large well ventilated stalls, bedded on wood shavings and fed ensiled grass. After 8 weeks in this environment, horses were confirmed to be free of pulmonary disease by clinical examination, arterial blood gas analyses and cytological examination of bronchoalveolar lavage fluid (BALF). Horses were confirmed to be susceptible to heaves by exposing them to an 8 h natural hay/straw challenge in a smaller, poorly ventilated stall bedded on straw and fed poorly saved, mouldy hay.

Respiratory disease was assessed clinically, prior to and after challenge, and assigned a clinical score from 0 to 10. These were computed by assessment of categorical and continuous clinical data: respiratory rate, presence of cough or nasal discharge, severity (mild, moderate or severe) of dyspnoea, presence of abnormal tracheal respiratory sounds and the presence and severity of abnormal lung sounds. We have demonstrated previously that the dyspnoea detected during a standard clinical examination protocol correlates with the maximal change in intrapleural pressure [10]. Carotid

arterial blood was collected and analysed for PaO_2 , $PaCO_2$ and pH in a Corning 238 Blood Gas Analyser (Ciba Corning Diagnostics Ltd., Halstead, UK). BALF was collected transendoscopically via a 2 m video-endoscope (Olympus CF 200HL, Olympus Keymed, Southend-on-Sea, UK). Horses were sedated with a combination of i.v. romifidine (30 µg/kg) and butorphanol (20 µg/kg). The volume of tracheal secretions was scored during endoscopic inspection at the level of the thoracic inlet on a scale of 0–5 [10]. BAL was performed by instillation of 300 mL (37 °C) 0.9% NaCl and aspirated using a 60 mL syringe. BALF cell viability was assessed by trypan blue exclusion. Total and differential BALF cell counts were performed using a haemocytometer and Leishmann's-stained cytocentrifuge preparations [11].

These preliminary studies demonstrated that all horses were free of pulmonary disease prior to challenge (clinical score ≤ 1 , normal blood gas analysis, tracheal secretions $\leq$ grade 1 and BALF neutrophil ratio $\leq 5\%$) and all developed signs of significant respiratory dysfunction (median [range] clinical score 3.5 [3–5]), arterial hypoxemia and airway inflammation (median BALF neutrophil ratio 61.8% [21.9–89.9]) following challenge. Previous work in this laboratory has demonstrated that hay/straw challenge does not induce lung dysfunction or inflammation in normal (non-susceptible) horses [6].

After at least 8 weeks in a controlled environment, horses were again confirmed to be free of pulmonary disease (baseline evaluation). In addition, blood was collected for haematological analysis and assessment of peripheral neutrophil function, BALF cells were harvested for baseline cytology, assessment of cell function and immunohistochemical studies and BALF supernatant was used for measurement of neutrophil elastase type 2A (ENE2A) content. Ten days later horses were exposed to a 5 h hay/straw challenge. Immediately prior to challenge (0 h) clinical scores were computed and jugular venous and carotid arterial blood samples collected for haematological and arterial blood gas analysis. Clinical scoring, venous and arterial blood collection, tracheal secretion scoring and BALF collection were then repeated at 5 and 24 h and 4, 7 and 14 days after the initiation of the challenge.

To ensure that any localized inflammatory response induced by prior bronchoscopy and BALF collection would not confound subsequent data, BALF was collected from anatomically discrete lung segments at all time-points less than 7 days apart [12]. A complete sham challenge with bronchoscopy and BALF collection at 0 and 19 h and 4, 7 and 14 days was also performed in all horses; this demonstrated that sequential BAL alone had no significant effect on clinical scores, BALF recovery, BALF cell viability or absolute or differential BALF cell counts in comparison with values at 0 h (data not shown). Moreover, sequential BAL had no effect on either blood neutrophil numbers or peripheral blood neutrophil or BALF cell function as assessed by agonist-stimulated chemiluminescence (data not shown).

Isolation of peripheral blood neutrophils

Equine peripheral blood neutrophils were prepared using discontinuous plasma/Percoll gradients [13]. These cells were washed sequentially in autologous platelet-poor plasma (PPP) (500 g, 6 min), phosphate-buffered saline (PBS) without Ca^{2+}

and Mg^{2+} /0.1% bovine serum albumin (BSA)/10 mM HEPES, pH 7.4 (235 g, 6 min) and PBS/0.1% BSA/10 mM HEPES, pH 7.4 (235 g, 6 min) prior to suspension in PBS. This method generates equine neutrophils with high cell viability/purity and minimal cell priming [13].

Assessment of constitutive apoptosis in isolated peripheral blood neutrophils

Freshly isolated peripheral blood neutrophils were suspended at $5 \times 10^6/\text{mL}$ in Iscove's modified Dulbecco's medium supplemented with 10% autologous serum and 100 U/mL penicillin and 100 U/mL streptomycin. Neutrophils were aged in flat-bottomed polypropylene flexiwells at 37°C in a humidified 5% CO_2 atmosphere for 20 h. To study the effect of any soluble components of BALF on the rate of constitutive apoptosis, neutrophils were cultured with 10% (v/v) BALF supernatant for 20 h. Neutrophil apoptosis was assessed morphologically [14]. In these experiments neutrophil viability after 20 h was $99.0 \pm 0.4\%$ and did not vary with any experimental condition. Ultrastructural and DNA electrophoresis studies supported the assertion that cells with characteristic darkly staining pyknotic nuclei under light microscopy were apoptotic rather than necrotic (data not shown).

Processing of bronchoalveolar lavage fluid

Recovered BALF was transported on ice and filtered through $60\mu\text{m}$ sterile nylon mesh; this did not affect absolute or differential BALF cell counts. Aliquots were taken to determine cell viability, absolute and differential cell counts and the proportion of neutrophils with apoptotic morphology. BALF cells were pelleted (340 g, 6 min) and the supernatant decanted, centrifuged (1800 g, 10 min) and stored at -70°C . BALF cells were then washed twice (PBS without Ca^{2+} and Mg^{2+} /0.1% BSA/10 mM HEPES, pH 7.4 and PBS/0.1% BSA/10 mM HEPES, pH 7.4, 235 g, 6 min) prior to re-suspension in PBS for chemiluminescence studies ($12.5 \times 10^6/\text{mL}$) or PBS/0.1% BSA, pH 7.4 for immunohistochemistry ($0.5 \times 10^6/\text{mL}$).

Assessment of neutrophil respiratory burst activity

Basal, fMLP ($1\mu\text{M}$) and PMA (100 ng/mL)-stimulated respiratory burst activity in peripheral blood neutrophils was measured by lucigenin-dependent chemiluminescence (Luci-DCL) using a ML 3000 microtitre plate luminometer (Dynex Technologies Ltd., Billingshurst, UK) as described previously [13]. Luci-DCL has been demonstrated to provide a sensitive and selective measurement of extracellular superoxide anion release [15]. In contrast, the respiratory burst activity of neutrophils within the mixed population of airspace cells harvested by BAL was assessed by luminol-dependent chemiluminescence (Lum-DCL). The chemiluminescence activity of phagocytes in the presence of the luminogenic probe luminol is almost entirely dependent upon the haemoprotein myeloperoxidase [16, 17] found in the azurophilic granules of neutrophils and the lysosomes of monocytes [18]. Studies of human alveolar macrophages mixed with peripheral blood neutrophils *in vitro* [19] and BALF cells from human patients with inflammatory lung

diseases [19–21] have demonstrated that for human BALF cells Lum-DCL almost exclusively reflects neutrophil respiratory burst activity. This was tested specifically for equine BALF cells by measurement of the basal- and agonist-stimulated Lum-DCL of both neutrophilic BALF harvested from heaves-affected horses and following addition of allogenic peripheral blood neutrophils to normal ($<5\%$ neutrophils) BALF to produce samples containing 0–90% blood neutrophils (10% steps) while maintaining a constant cell number. This demonstrated a highly significant and linear relationship ($r^2 = 0.81$, $P < 0.0001$, $n = 4$). Analysis of the relationship between basal and agonist-stimulated Lum-DCL and the proportion of neutrophils in BALF (Spearman's Rank correlation coefficients) also demonstrated a significant linear relationship (basal, $r = 0.70$, $P < 0.0001$; fMLP-stimulated Lum-DCL, $r = 0.76$, $P < 0.0001$; PMA-stimulated Lum-DCL, $r = 0.78$, $P < 0.0001$; $n = 36$). These data suggested that Lum-DCL can be used as an index of the functional responsiveness of neutrophils contained within the mixed population of cells harvested by BAL. Measurement of Lum-DCL of BALF cells was performed as detailed above with the exception that luminol (1 mM in PBS/0.1% BSA, pH 7.4) replaced lucigenin. Attempts to physically separate neutrophils from BALF by methods including continuous density gradient centrifugation, flow cytometric light scattering characteristics and positive or negative immunological selection were unrewarding (data not shown).

To ensure that BALF neutrophil function was not affected by endoscopic BALF collection, *in vitro* sham lavage experiments were performed using peripheral blood neutrophils prepared from three normal horses. Two aliquots of freshly prepared neutrophils were re-suspended in 0.9% NaCl ($0.5 \times 10^6/\text{mL}$). One aliquot was maintained on the bench at room temperature and the other was aspirated through the endoscope using a 60 mL syringe. Cells were washed twice and prepared for chemiluminescence studies as described above. There was no significant difference in either basal or fMLP-stimulated Luci- or Lum-DCL between the control and sham lavage cells (data not shown).

Measurement of equine neutrophil elastase type 2A bronchoalveolar lavage fluid

ENE2A was measured in BALF supernatant by specific ELISA as described previously [22]. To ensure that ENE 2A release from neutrophils was not induced by the trauma of endoscopic BALF collection, ENE 2A was measured in cell-free supernatants prepared after the *in vitro* sham lavage procedure described above. Only small amounts ($<10\text{ ng/mL}$) of ENE 2A were detectable in the supernatants of sham lavage or control cells and these were not significantly different (data not shown).

Ultrastructural and immunohistochemical studies

To aid identification of apoptotic neutrophils existing both free in BALF and contained within phagocytic alveolar macrophages, BALF cells harvested from horses 24 h after challenge were prepared for transmission electron microscopy. After washing, 40×10^6 cells were pelleted (230 g, 6 min), re-suspended and fixed (sodium cacodylate buffer

(0.1 M, pH 7.4)/2.5% glutaraldehyde, then 1% osmium tetroxide in sodium cacodylate buffer, 45 min). After dehydration and infiltration in araldite CY212/dodecenyl succinic acid, samples were hardened (67% dibutylphthalate/33% dimethylaminomethylene). Uranyl acetate/lead citrate-stained sections were examined and imaged (Philips EM400, Philips Electron Optics, Eindhoven, the Netherlands).

To confirm that the densely staining chromatin remnants recognized in alveolar macrophages on Leishmann's-stained preparations represented apoptotic nuclear chromatin, cyto-centrifuge preparations of BALF harvested 24 h after challenge were prepared on 3-aminopropyltriethoxysilane (TESPA)-coated slides, fixed (4% formalin, 10 min) and stained by the terminal deoxynucleotide transferase-mediated UTP nick end-labelling (TUNEL) method. This was performed using a FragEL™ DNA fragmentation detection kit (Calbiochem-Novabiochem, Nottingham, UK) according to the manufacturers' instructions.

At each time-point, cyto-centrifuge preparations of BALF cells were made on TESP-coated slides, fixed and stained for ENE 2A as previously described [22]. At each time-point 500 alveolar macrophages were examined on duplicate slides and those containing intracellular ENE 2A-positive neutrophil remnants counted. Alveolar macrophages were only scored as positive if ENE 2A staining was associated with densely staining intra-cytoplasmic nuclear material consistent with apoptotic chromatin or formed tight clusters consistent with engulfment of an entire cell.

Statistical analysis

Data are reported as median and (range) values for *n* independent experiments. Paired and unpaired analyses of these data were performed using the Wilcoxon's Sign Rank and Mann-Whitney tests, respectively. Correlations were examined using the Spearman's Rank Sign test or when one variable was controlled, linear regression coefficients were calculated by least squares analysis. Multiple groups were compared by the Kruskal-Wallis test. Within group analyses of data from these *in* and *ex vivo* studies were performed by comparison of data from each post-challenge time-point with that collected at baseline. Analyses were performed using Minitab (Minitab Inc., State College, PA, USA) and GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Results were considered significant at the 5% level.

Results

Clinical effects of hay/straw challenge in heaves-susceptible horses

Hay/straw challenge induced a significant increase in the clinical score that was evident immediately post-challenge (5 h) and remained elevated at 24 h and 4 days after exposure (Fig. 1a). The presence of abnormal respiratory sounds audible over the distal trachea and lung fields (6/6 horses at 5 and 24 h) and increased abdominal expiratory effort (5/6 horses at 5 and 24 h) were the most consistent clinical indicators of lung disease. Significant arterial hypoxaemia was evident at 5 and 24 h post-challenge (Baseline PaO_2 104

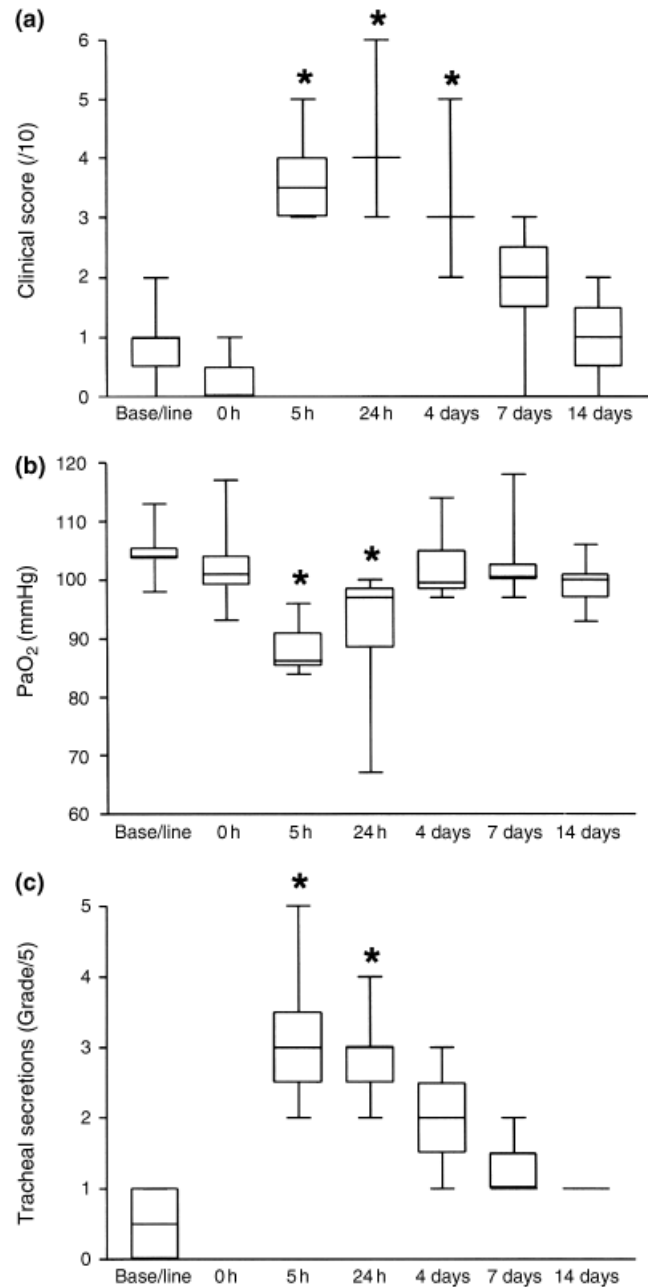


Fig. 1. Effect of hay/straw challenge on clinical scores, PaO_2 and volume and nature of tracheal secretions in susceptible horses. Horses ($n = 6$) were exposed to hay/straw for 5 h (0–5 h) and (a) clinical scores, (b) PaO_2 and (c) tracheal secretions quantified at the time-points indicated. Baseline values (B/line) represent data collected from the same animals 10 days prior to experimental challenge. Bronchoscopic examination of tracheal secretions was not performed immediately prior to challenge (0 h). Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values (*represents $P < 0.05$ compared with baseline values).

(98–113) mmHg, 5 h PaO_2 86 (84–95) mmHg, 24 h PaO_2 97 (67–100) mmHg) (Fig. 1b). Arterial pH and $PaCO_2$ values remained normal at all time-points (data not shown). All animals developed copious, non-viscous mucopurulent secretions (bronchoscopic grade 2 or greater) at 5 and 24 h post-challenge compared with 4/6 animals with grade 1 and 2/6 animals with no visible secretions at baseline (Fig. 1c). After

24 h the volume of tracheal secretions decreased progressively with all animals having grade 1 secretions by day 14.

Effects of hay/straw challenge on peripheral blood neutrophil number and function

There was a small but significant increase in the circulating leucocyte numbers at 24 h post-hay/straw challenge reflecting a rise in the total neutrophil count (Table 1). The priming and activation status of peripheral blood neutrophils was assessed by determining the capacity of these cells to (i) generate reactive oxygen intermediates under basal, fMLP and PMA-stimulated conditions and (ii) undergo spontaneous apoptosis when aged *in vitro*. Spontaneous respiratory burst activity, monitored by Luci-DCL, in unstimulated cells was minimal and not affected by hay/straw challenge (Table 1). By contrast, both fMLP- and PMA-stimulated Luci-DCL increased significantly in neutrophils isolated 24 h post-challenge but returned to baseline levels by 4 days (Table 1). This ability of the hay/straw challenge stimulus to modulate fMLP-stimulated Luci-DCL in peripheral blood neutrophils is illustrated in Fig. 2. In addition, circulating neutrophils isolated at 5 and 24 h post hay/straw challenge demonstrated a lower rate of spontaneous apoptosis when aged for 20 h *in vitro* (Table 1). This parameter has been shown to be a sensitive indicator of neutrophil priming status in human cells [23]. These data, together with our previous observation of an up-regulation in secretagogue-induced respiratory burst activity in isolated equine neutrophils following LPS or TNF- α incubation [13], suggest that hay/straw challenge induces significant priming of peripheral blood neutrophils in addition to a transient neutrophilic leukocytosis.

Characterization of the airspace response to hay/straw challenge

As detailed above, serial BAL of sham exposed susceptible horses had no effect on absolute or differential cell counts (or basal or agonist-stimulated Lum-DCL) in BAL samples obtained at time-points up to 14 days (data not shown). The

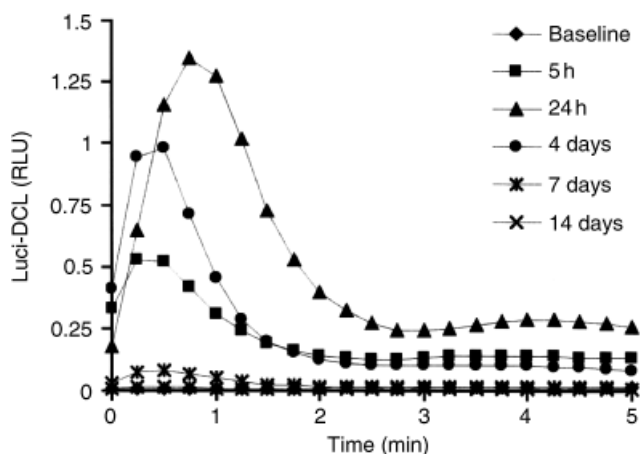


Fig. 2. Upregulation of fMLP-stimulated lucigenin-dependent chemiluminescence in isolated peripheral blood neutrophils following hay/straw challenge. Time-course of fMLP-stimulated (1 μ M) Luci-DCL in peripheral blood neutrophils isolated at the time-points indicated following hay/straw challenge. Individual data points represent the mean of triplicate determinations obtained from a single animal representative of five others.

Table 1. Effects of hay/straw challenge on peripheral blood leucocyte numbers and function

	Baseline	0 h	5 h	24 h	4 days	7 days	14 days
Total leucocyte count ($\times 10^9/L$)	7.7 (5.4–11.7)	8.2 (5.4–10.3)	8.1 (5.4–9.7)	10.4* (9.6–13.7)	8.8 (5.5–19.5)	9.6 (5.2–11.1)	9.6 (6.8–11.6)
Neutrophil count ($\times 10^9/L$)	4.3 (2.4–8.9)	5.4 (2.6–6.4)	5.6 (3.4–7.5)	8.4* (6.5–10.3)	5.0 (2.7–6.3)	5.6 (3.1–7.9)	6.3 (3.4–8.5)
Basal Luci-DCL (RLU)	1.1 (0.4–1.8)	ND	0.5 (0.4–1.5)	2.0 (0.4–1.5)	3.3 (0.6–4.8)	0.6 (0.4–13.6)	0.7 (0.2–1.6)
fMLP-stimulated Luci-DCL (RLU)	3.7 (0.1–5.2)	ND	5.2 (0.2–8.3)	10.8* (4.8–31.2)	8.6 (0.6–35.5)	2.1 (0.4–11.6)	0.5 (0.1–3.0)
PMA-stimulated Luci-DCL (RLU)	181.9 (73.3–307.9)	ND	113.4 (96.6–182.2)	415.6* (130.4–509.0)	215.0 (131.7–612.2)	173.7 (133.5–277.7)	134.0 (99.4–257.2)
Susceptibility to apoptosis (%)	56.2 $\pm$ 4.7	ND	44.0 $\pm$ 7.1*	43.4 $\pm$ 3.9*	48.6 $\pm$ 6.1	58.1 $\pm$ 7.1	57.5 $\pm$ 6.2

Total and differential leucocyte counts were performed on samples of jugular venous blood taken at the time-points indicated prior to and following hay/straw challenge. Lucigenin-dependent chemiluminescence was quantified in purified neutrophils under non-stimulated (basal), fMLP and PMA-stimulated conditions. The capacity of isolated neutrophils to undergo spontaneous apoptosis during 20 h in culture *in vitro* was determined at each time-point. Baseline values (B/line) represent data obtained from the same horses 10 days prior to experimental hay/straw challenge. Data represent median (range) values for six animals with each observation being the mean of triplicate determinations.

*Values in bold type indicate $P < 0.05$ compared with baseline values.

recovery of BALF from animals 10 days prior to hay/straw challenge (baseline) was 46 (38–63)% of the volume instilled and did not vary significantly with any experimental condition or time-point studied. Likewise, the viability of BALF cells at baseline was 96.6 (93.6–100)% and remained at or above this level at all time-points studied.

BALF was collected in all animals 10 days prior to hay/straw challenge (baseline) and immediately following (5 h) and at 1, 4, 7 and 14 days. Total BALF cell counts were significantly elevated at 5 h post-challenge (Table 2). This reflected the very substantial increase in absolute neutrophil numbers harvested from the distal airspaces at 5 h and 24 h. Of note, there was a modest but consistent decrease in absolute macrophage ($P < 0.05$ at 5 h, 1 and 7 days) and lymphocyte numbers ($P < 0.05$ at 1 day) post-challenge (Table 2) but no change in mast cell, basophiloid cell or epithelial cell number (all $< 0.02 \times 10^5/\text{mL}$) (data not shown). No eosinophils were identified in BALF samples. The above macrophage data are consistent with previous airway challenge studies and may reflect the selective effects of airway and lung inflammation on macrophage recovery [24].

The proportion of neutrophils in the BALF increased from a median of 2.2 (1.0–4.8)% at baseline to 61.8 (14.3–89.9)% 5 h post-challenge. Thereafter, the % BALF neutrophils declined rapidly, reaching baseline values by 7 days (Fig. 3a). Figure 3b shows the tight temporal association between the differential neutrophil count in the serial BALF samples and the concentration of ENE 2A in lavage supernatants, which remained significantly elevated to day 4 post-challenge. This suggests active degranulation of the neutrophils recruited after hay/straw challenge.

To assess the functional and priming status of the airspace neutrophils, fMLP-stimulated Lum-DCL was measured at each time-point. Figure 3 shows the large time-dependent increase in fMLP-stimulated Lum-DCL in BALF cells recovered at 5 and 24 h post-challenge. Of note, comparison of the magnitude of the luminol (and hence neutrophil-dependent) CL response to fMLP in BALF cells with that obtained in an identical number of peripheral blood neutrophils indicated that BALF neutrophils had a 3–5-fold greater capacity to generate reactive oxygen intermediates compared with peripheral blood cells. Although the number of neutrophils in the BALF from non-challenged animals was much lower ($0.2 [0.1–0.6] \times 10^5/\text{mL}$ compared with $7.7 [2.3–57.7] \times 10^5/\text{mL}$ 5 h post-challenge), a similar proportional increase was observed in fMLP-stimulated Lum-DCL. These data suggest that neutrophil migration into the airspaces under either challenged or non-challenged conditions causes a similar degree of neutrophil priming.

Detection of neutrophil apoptosis and phagocytosis of apoptotic neutrophils in BAL samples following hay/straw challenge and the relationship of these events to the resolution of airway inflammation

The rapid but transient nature of the airspace neutrophilic response to hay/straw challenge suggested the involvement of an active clearance mechanism for effete or aged cells. To determine the potential role of neutrophil apoptosis in the resolution phase, more detailed cytological and immunohistochemical examinations of the BALF cells were performed. Small numbers of neutrophils displaying typical apoptotic morphology were identified on cytocentrifuge preparations and transmission EM (Fig. 4). However, this number increased significantly at 1 and 4 days post-challenge (median [range] number of apoptotic neutrophils $\times 10^4/\text{mL}$ in cytocentrifuge preparations: baseline 0 [0–0.23]; 1 day 0.14 [0.03–5.40]; 4 days 0.17 [0.03–0.7]); the values at 5 h and 7 and 14 days were not significantly different from baseline values (data not shown).

Examination of cytocentrifuge preparations also identified a population of alveolar macrophages that contained densely staining intra-cytoplasmic chromatin bodies consistent with having phagocytosed apoptotic neutrophils (Fig. 5a). The appearance of these remnants varied from being clearly identifiable as entire apoptotic neutrophils to grouped clusters of condensed chromatin. Confirmation that these structures represented apoptotic neutrophils was obtained by transmission EM, TUNEL staining and immunohistochemistry using ENE 2A antibodies (Figs. 5b–d). The pattern of ENE 2A staining appeared very similar to previous reports of neutrophil myeloperoxidase staining within human BALF macrophages [25]. The presence of tight clusters of intense ENE 2A staining without associated densely staining chromatin remnants suggests that the ENE 2A remains detectable within the alveolar macrophage some time after the destruction of visible nuclear chromatin.

To examine the temporal relationship between the self-limiting airspace neutrophilia and the appearance of neutrophil apoptosis and engulfment, ENE 2A positive macrophages were enumerated in a blinded manner at each time-point. The number of ENE 2A positive macrophages rose sharply at 1 day post-challenge and remained significantly elevated at 4 days (Fig. 6). Critically, the number of ENE 2A positive macrophages was maximal at the time (1 day) when neutrophil numbers were declining most rapidly (Fig. 6b).

Finally, examination of the effect of BALF supernatants on the rate of spontaneous apoptosis of isolated blood neutro-

Table 2. Total and absolute cell counts in BALF collected from susceptible horses following hay/straw challenge

Cell type	Baseline	5 h	24 h	4 days	7 days	14 days
Total ($\times 10^5/\text{mL}$)	8.0 (4.9–13.1)	14.2* (10.5–64.2)	6.8* (5.1–17.1)	5.2 (3.8–7.9)	5.8 (2.5–6.6)	7.7 (4.6–11.1)
Neutrophil ($\times 10^5/\text{mL}$)	0.2 (0.1–0.6)	7.7* (2.3–57.7)	1.5* (1.1–12.0)	0.3 (0.2–0.6)	0.2 (0.1–0.4)	0.2 (0.1–6.5)
Macrophage ($\times 10^5/\text{mL}$)	3.7 (2.2–6.2)	2.0* (1.7–3.4)	1.5* (1.4–2.8)	2.1 (1.2–3.3)	1.8* (1.5–2.6)	3.4 (1.9–3.9)
Lymphocyte ($\times 10^5/\text{mL}$)	4.1 (2.4–6.7)	3.8 (2.4–6.7)	2.5* (1.7–3.6)	2.3 (1.7–4.1)	3.1 (0.8–4.7)	3.9 (2.5–7.3)

Bronchoalveolar lavage fluid (BALF) was collected 10 days before (Baseline: B/line) and at the time-points indicated after hay/straw challenge. Total nucleated and absolute cell counts were calculated in duplicate on raw BALF using a haemocytometer and derived from differential cell counts of Leishman's stained cytocentrifuge preparations. Data represent median (range) values for $n = 6$ animals with each observation being the mean of triplicate determinations.

*Values in bold type indicate $P < 0.05$ compared with baseline values.

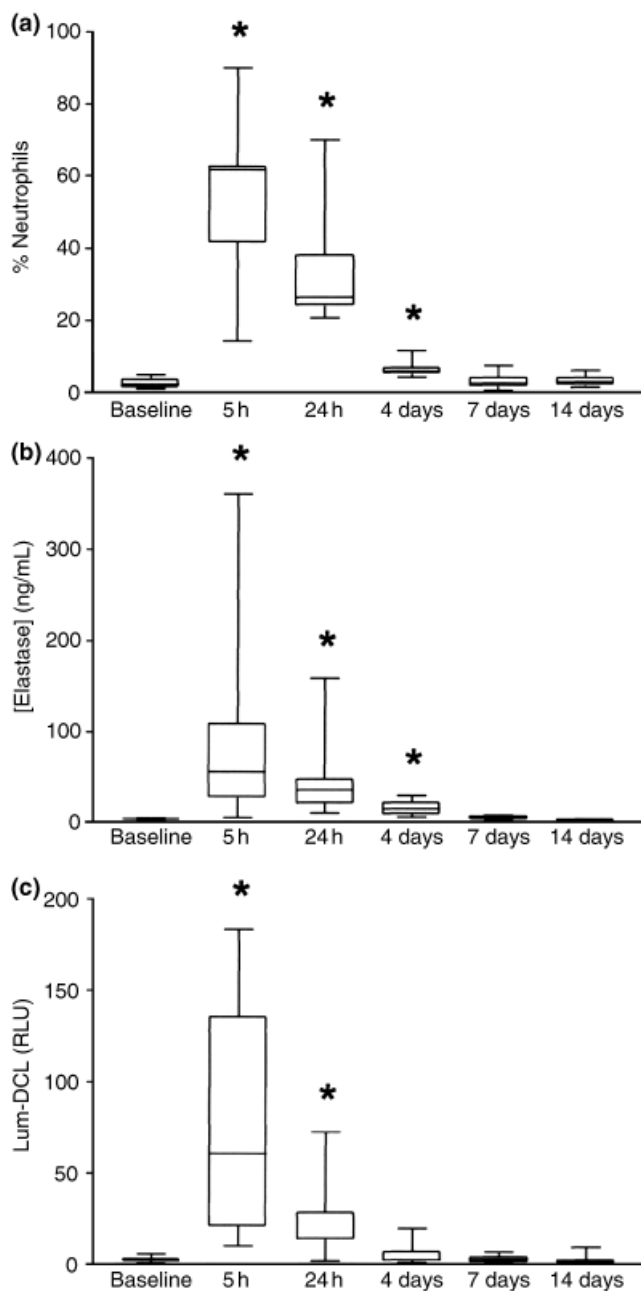


Fig. 3. Effect of hay/straw challenge on neutrophil differential cell counts, equine neutrophil elastase type 2A and neutrophil function in bronchoalveolar lavage fluid (BALF) in susceptible horses. BALF was collected 10 days before (baseline) and at the time-points indicated after hay/straw challenge. BALF differential neutrophil counts (a) were determined in duplicate on Leishman's stained cytocentrifuge preparations. Equine neutrophil elastase type 2A (ENE 2A) content in BALF supernatant (b) was measured by specific ELISA using purified ENE 2A as a standard. fMLP-stimulated ($1 \mu\text{M}$) Lum-DCL (c) was quantified over a 5 min time-course as detailed in the methods. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values (*represents $P < 0.05$ compared with baseline values, $n = 6$).

phils *in vitro* failed to reveal any soluble pro-apoptotic factor at any of the time-points examined. Indeed, a small but significant survival effect was observed when neutrophils were incubated with BALF supernatants obtained at 5 h and 1 day post-challenge (Fig. 7).

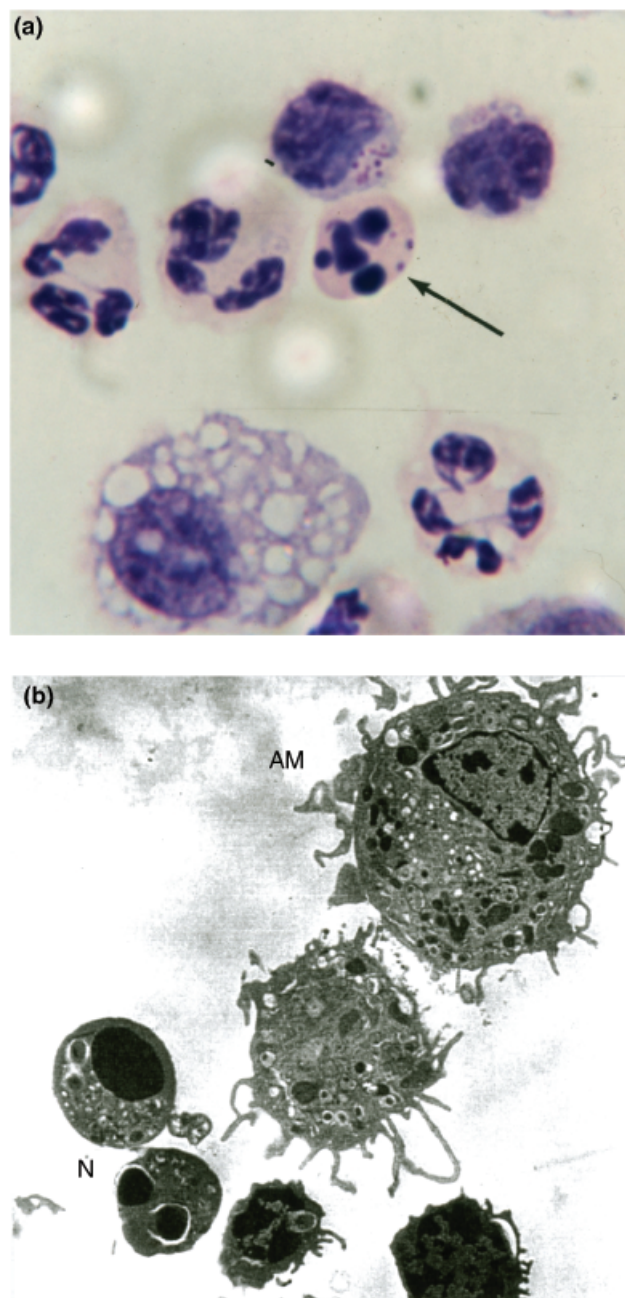


Fig. 4. Detection of apoptotic neutrophils in bronchoalveolar cell populations following hay/straw challenge. Bronchoalveolar lavage fluid (BALF) was collected 24 h after hay/straw challenge and examined by (a) light microscopy (magnification $\times 1240$) on Diff-Quik-stained cytocentrifuge preparations. An apoptotic neutrophil is marked (arrow), note densely staining pyknotic chromatin fragments (b) transmission electron microscopy (magnification $\times 3730$). Neutrophils displaying typical apoptotic morphology are marked (N). Note condensed nuclear chromatin and retention of intracellular granules (AM, alveolar macrophage).

Discussion

We have demonstrated the rapid kinetics of neutrophil accumulation and removal in the airspaces of heaves-susceptible horses following exposure to mouldy hay and straw. Implicit to this response, circulating neutrophil numbers increased and the respiratory burst activity of these

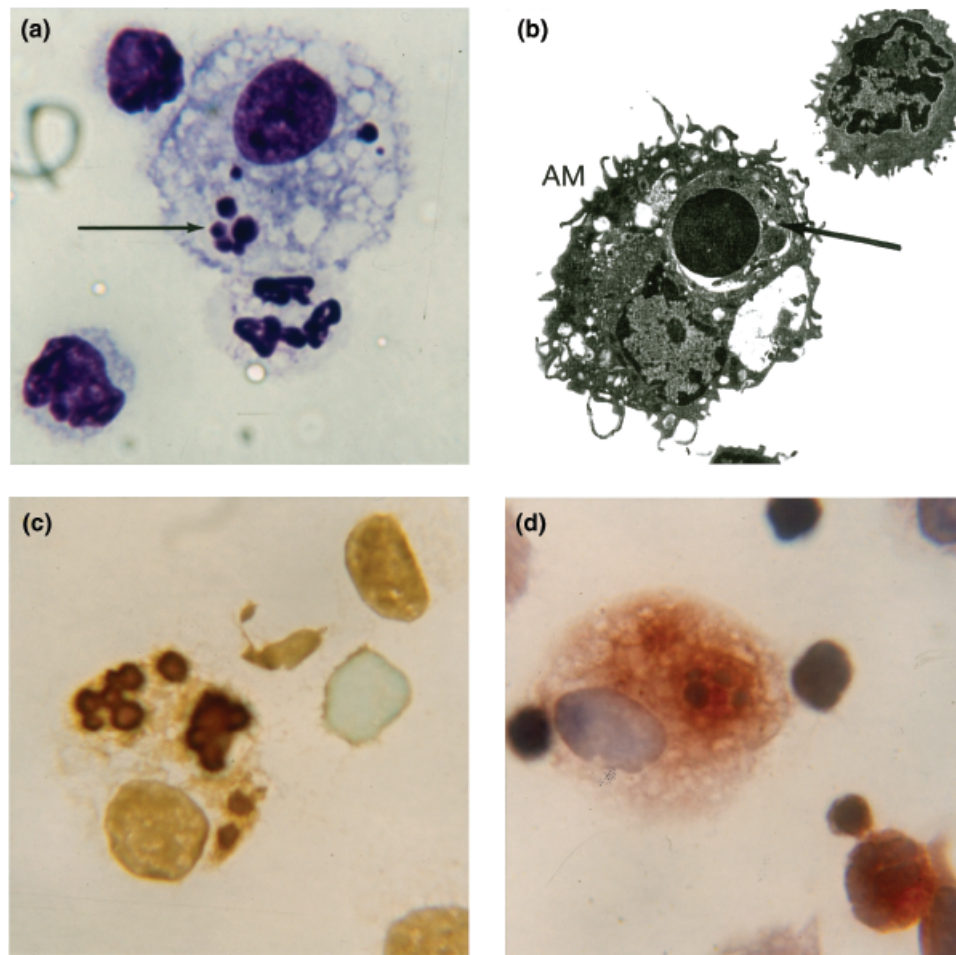


Fig. 5. Identification of apoptotic neutrophils within alveolar macrophages following hay/straw challenge. Bronchoalveolar lavage fluid (BALF) was collected 24 h after hay/straw challenge and examined by (a) light microscopy (magnification $\times 1300$) on Diff-Quik-stained cytocentrifuge preparations. An apoptotic neutrophil contained within an alveolar macrophage is marked (arrow). (b) Transmission electron microscopy (magnification $\times 4800$). An alveolar macrophage (AM) has engulfed an intact apoptotic neutrophil (arrow). (c) Light microscopy (magnification $\times 1850$) after terminal deoxynucleotidyl transferase-mediated UTP nick end-labelling (TUNEL) staining of cytocentrifuge preparations. An alveolar macrophage contains several clusters of TUNEL positive apoptotic bodies. TUNEL labeling detected with DAB (brown), slides counterstained with methyl green. (d) Light microscopy (magnification $\times 1330$) after immunohistochemical staining for ENE 2A on cytocentrifuge preparations. Alveolar macrophage containing densely staining pyknotic bodies co-localized with ENE2A staining. ENE2A detected with AEC (red), slides counterstained with Mayer's haematoxylin.

cells as well as those recruited to the distal airways was markedly enhanced. Furthermore, evidence has been presented to support a significant role for neutrophil apoptosis and subsequent macrophage phagocytosis in the rapid clearance of the airway neutrophil load.

In this naturally occurring disease, susceptible animals experience recurrent episodes of airway inflammation followed by complete remission of pulmonary disease after removal of the provocative stimulus [6]. This provided us with a unique and naturally occurring model of spontaneously resolving neutrophil-mediated pulmonary inflammation.

Currently the precise aetiology of heaves remains undefined. However, results of specific antigen inhalation challenges and provocative immunological testing suggest that the disease represents a pulmonary hypersensitivity response to inhaled mould antigens and other environmental contaminants [6, 7]. The characterization of an environmental lung disease that results in recurrent episodes of airway inflammation and obstruction has led to its proposal as an appropriate animal model for human asthma [26, 27] and the recent

demonstration of a T helper type 2 cytokine profile in inflammatory cells of affected horses further supports an allergic aetiology [28].

Our group and others have reported extensively on the aerobiology of mouldy hay-straw and have identified a plethora of materials including bacteria and their component endotoxin, fungal particles (including spores, hyphae and mycotoxins), noxious gasses (e.g. ammonia and hydrogen sulfide), forage mites, animal-derived material (e.g. hair and faeces) and plant fragments [29]. Moreover, spores of approximately 70 species of moulds and actinomycetes have been identified all with sufficiently small diameter ($<5\mu\text{m}$) to be respirable into the distal small airways and alveoli. These data have been supported by inhalation challenges with extracts of the fungus *A. fumigatus* and the thermophilic actinomycete *F. rectivirgula*.

In contrast to previous reports [30] this study has demonstrated a close temporal association between the airway neutrophilia and clinical assessment of pulmonary dysfunction. This agrees with work demonstrating a close

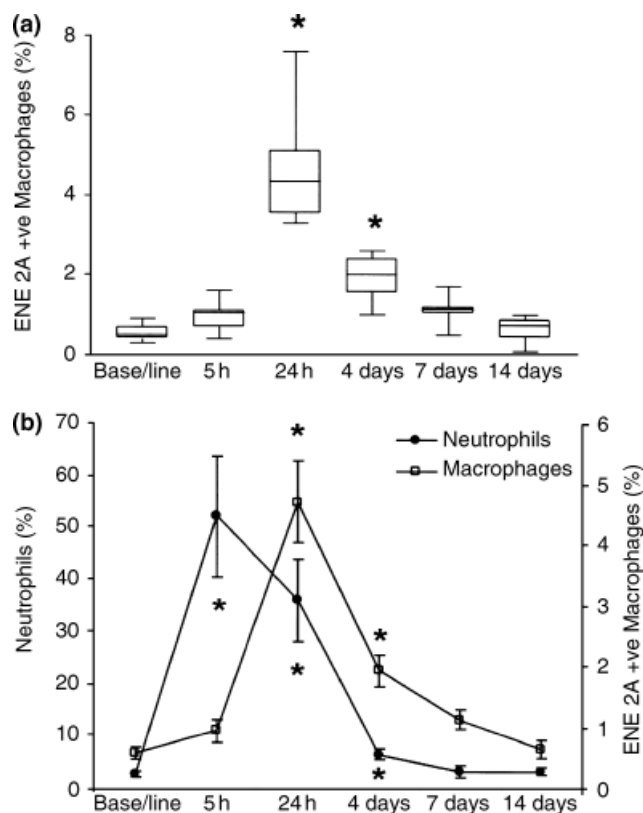


Fig. 6. Kinetics of macrophage clearance of apoptotic neutrophils *in vivo*. Bronchoalveolar lavage fluid (BALF) was collected 10 days before (Base-line) and at the time-points indicated after hay/straw challenge. (a) Cytocentrifuge preparations were examined immunohistochemically for the presence of ENE 2A. At each time point 500 alveolar macrophages were examined on duplicate slides and the proportion containing ENE 2A positive cell remnants recorded. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values (*represents $P < 0.05$ compared with baseline values, $n = 6$). (b) BALF differential neutrophil counts were determined in duplicate on Leishman's stained cytocentrifuge preparations. The figure demonstrates the relationship between the proportions of neutrophils and ENE 2A positive alveolar macrophages in BALF samples collected at each time-point. For improved clarity the data are presented as mean $\pm$ SEM values ($n = 6$).

kinetic relationship between the onset of pulmonary dysfunction in this model and the up-regulation of pro-inflammatory mediators likely to be important in neutrophil recruitment, namely nuclear factor- κ B and intercellular adhesion molecule-1 [26]. The diffuse nature of the inflammatory response [31] also permitted us to obtain repetitive and representative BALF collections that circumvented the problems of the confounding local and systemic BAL-associated inflammatory response recognized in many other species [32–34]. Likewise, although the early (7 h) post-challenge kinetics of neutrophil recruitment to the lungs of susceptible horses has been investigated scintigraphically [30], to the authors' knowledge the time-course of neutrophil accumulation and clearance in the airspaces has not been documented.

We have previously shown that exposure of equine neutrophils to well described human priming agents (LPS, platelet-activating factor and tumour necrosis factor- α) causes cell shape change and primes them for an enhanced respiratory burst response upon subsequent secretagogue activation [13]. Moreover, we have demonstrated that priming is a pre-requisite for functional coupling of fMLP

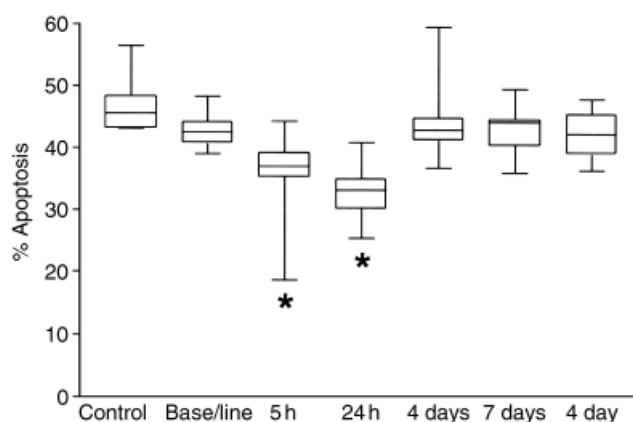


Fig. 7. Effect of bronchoalveolar lavage fluid (BALF) supernatant on the rate of spontaneous apoptosis of equine peripheral blood neutrophils. Equine peripheral blood neutrophils isolated from healthy horses (5×10^6 /mL) were incubated in Iscove's modified Dulbecco's medium (supplemented with 10% autologous serum) at 37 °C for 20 h in the presence of 10% normal NaCl (control) or 10% BALF supernatant collected 10 days before (Baseline) and at the time-points indicated after hay/straw challenge of susceptible horses. After 20 h in culture, the cells were resuspended and the percentage apoptosis assessed morphologically on Diff-Quik-stained cytocentrifuge preparations. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values (*represents $P < 0.05$ compared with baseline values, $n = 6$).

receptors in equine neutrophils [13]. Hence the absence of significant respiratory burst activity in un-primed equine neutrophils when stimulated with fMLP suggests that this assay can be used as an index of functional priming *in vivo*. The appearance of significant fMLP-stimulated respiratory burst activity in peripheral blood neutrophils 1 day after hay/straw challenge provided compelling evidence of *in vivo* priming. Similarly, Olszewski et al. [35] detected an enhancement in serum-treated zymosan-stimulated superoxide anion generation in blood neutrophils isolated from heaves-susceptible horses following challenge. Another priming-related phenomenon, enhanced β_2 -integrin-dependent adhesion has also been recognized 24 and 72 h post-challenge [36]. Primed peripheral blood neutrophils have also been demonstrated in the circulation of symptomatic human asthma patients [37, 38]. Primed circulating neutrophils have an increased transit time although the pulmonary microvasculature [39], being preferentially retained [40], because of changes in cell size and deformability [41, 42].

The reduction in the rate of constitutive apoptosis in peripheral blood neutrophils after challenge is likewise consistent with their primed state [23]. A similar response has been observed in blood neutrophils harvested from human patients with severe sepsis, burns and various other neutrophilic inflammatory diseases [43–45]. The appearance of a significant blood neutrophilia 1 day post-challenge is likely to reflect release of relatively immature cells from the bone marrow; while this would not account for the enhanced responsiveness of these cells to fMLP [13], it is possible that the presence of less mature cells may contribute to the slower rate of spontaneous apoptosis observed at 24 h. However, the anti-apoptotic effect of plasma obtained from burns patients was abrogated by a neutralizing antibody to GM-CSF [44] suggesting that systemic cytokine release may be a more significant determinant of neutrophil longevity *ex vivo*. The

paucity of currently available immunological reagents specific for equine TNF- α , IL-8, GM-CSF and other potentially relevant cytokines has hampered this and other studies on the systemic cytokine profile in equine heaves.

In heaves-susceptible horses, elimination of the inciting stimulus allows the return of lung function and airway inflammatory indices to baseline levels that are equivalent to non-susceptible animals [6] suggesting that complete remission is achievable. Moreover, histological studies suggest that the disease is primarily a neutrophilic 'endobronchiolitis' with minimal irreversible parenchymal pathology even in chronically affected animals [46].

The significant release of ENE2A into the airways and enhanced respiratory burst activity of BALF neutrophils demonstrates significant up-regulation of their functional activity upon recruitment to the airspaces following hay/straw challenge. However, comparison of blood and BALF neutrophil function pre- and post-challenge suggests that neutrophil priming/activation is intimately linked to the transmigration of the cells to the airways irrespective of the recruitment stimulus. Analysis of markers of neutrophil activation in both peripheral blood and BALF cells from normal human subjects and those with pneumonia showed similar differential results but demonstrated enhanced activation of BAL-derived neutrophils from patients with pneumonia [47]. Thus the level of activation of airway neutrophils is likely to be determined by both the nature and intensity of the challenge stimulus.

The rapid and transient nature of the airway neutrophilia suggests that the signals for neutrophil recruitment abate extremely rapidly. In general, the pulmonary neutrophil kinetics reported here are comparable with other models of self-limiting airway neutrophilia observed in response to grain dust in man [48] and mice [49], LPS in rodents [3, 49] and oleic acid in rats [4]. Other studies in heaves-susceptible horses have also reported more persistent airway neutrophilia following a more prolonged challenge [50].

The temporal co-incidence between the period of maximal neutrophil loss from the airways and the appearance of significant numbers of ENE2A positive alveolar macrophages implies a causative association. As alveolar macrophages may also take up free elastase, great care was taken to score cells as ENE2A positive only when associated with convincing evidence of apoptotic neutrophil engulfment. Hence it is unlikely that we have overestimated the importance of this potential neutrophil clearance route. The absolute numbers of apoptotic neutrophils free in BALF were very small at all time points ($<1.7 \times 10^3/\text{mL}$ or 0.3% of total cell numbers). Although there was a small but significant increase in the proportion of free apoptotic neutrophils at day 4 this index accounted for no more than 3.8% of the total neutrophil population at any time point implying that there is efficient coupling between neutrophil apoptosis and macrophage ingestion. Occasional macrophages contained more than ten apoptotic neutrophils suggesting that this is also a high capacity clearance mechanism.

The fall in absolute BALF macrophage numbers recovered between 5 h and 7 days may indicate that the full importance of this clearance pathway was underestimated. While this is likely to reflect activation-enhanced macrophage adhesion [24] and bronchiolar obstruction [7], it is possible that

ingestion of apoptotic neutrophils triggers either apoptosis of the alveolar macrophage itself [4] or stimulates their emigration from the airways, perhaps to draining lymph nodes [51, 52]. Whilst these data support the involvement of this pathway in the resolution of natural disease, its precise net contribution to neutrophil clearance relative to, for example, expulsion of neutrophil-rich tracheal secretions remains an important topic of future research.

We also demonstrated the presence of neutrophil survival factor(s) in the distal airways following challenge, consistent with both the cytokine-mediated anti-apoptotic effects of BALF from human ARDS patients [53] and the GM-CSF-dependent enhanced survival of lung granulocytes from heaves-affected horses [54]. However, in our kinetic study, such a survival signal was present in the airways even when neutrophil numbers were falling rapidly. The simultaneous presence of such opposing features suggests that regulation of neutrophil longevity is complex and may involve mechanisms beyond soluble factors in epithelial lining fluid and opens the way for potential therapeutic intervention [55].

In summary, this study represents the first characterization of the onset and resolution of neutrophilic airway inflammation in a relevant and natural disease model of airway inflammation. Our data suggest that local allergen challenge has a significant impact on peripheral neutrophil number and function and more importantly supports previous experimental work that neutrophil apoptosis and macrophage removal may play a key role in the resolution of neutrophilic inflammation.

Acknowledgements

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